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Award Number: DAMD17-97-1-7145

TITLE: 99mTC Alpha-Fetoprotein: A Novel, Specific Agent for the Detection of Human Breast Cancer

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REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Final (15 Jun 97 - 14 Jun 00)

4. TITLE AND SUBTITLE
99mTC Alpha-Fetoprotein: A Novel, Specific Agent for the Detection of Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7145

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Because adenocarcinoma of the breast expresses receptors for alpha-fetoprotein (AFP), we studied Tc-99m radiolabeled AFP and its sub domains as agents to detect breast cancer. The biodistribution of Tc-99m radiolabeled natural (full length) and recombinant domain III (DIII) of human AFP was compared to the clinical agents Tc-99m sestamibi and Tl-201 in a murine model of human breast cancer. Estrogen receptor positive (MCF-7, T47, MTW9A) and estrogen receptor negative (MDA MB-231, BT20) human breast cancer xenografts were grown subcutaneously in the lateral thorax region of immunosuppressed mice (CB-17 SCID). Quantitative comparisons of percent-injected dose per gram of tissue (%ID/gram) and tumor to thigh ratio (T/Th) were performed at 0-60 minutes and at 24 hours following injection. For most tumors, T/Th for AFP and DIII was significantly greater than T/Th for Tc-99m sestamibi and Tl-201. DIII and AFP had significantly higher %ID/gram than either Tl-201 or Tc-99m sestamibi when considered across all tumor types (BT20, MCF7, MDA, MTW9A, T47) at both 60 minutes and 24 hours. In the final year of the work we showed similar properties for a 12 amino acid peptide derived from AFP. The data suggests that localization of Tc-99m AFP in human breast cancer xenografts is initially rapid, increases with time, and is superior to Tc-99m sestamibi and Tl-201. Given its high uptake by breast cancer cells, its low non-tumor localization and its rapid renal excretion, these Tc-99m AFP preparations should be useful agents to detect human breast carcinoma.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Alpha-fetoprotein, Scintigraphic Imaging, Xenografts	38
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Bruce R Line MD 7/14/00

PI - Signature Date

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INTRODUCTION:

Subject of Research Work

It is estimated that in the United States, 182,000 new cases of breast cancer were diagnosed in women and 46,000 women died of the disease.(1) In addition to its cost of human lives, breast cancer consumes more health care dollars than any other cancer.(2) There is no question that early detection and removal of breast cancer is currently the best approach for curing this disease. Over 70% of US women in the age groups at risk for breast cancer have had at least one abnormal mammogram that detected a mass, a cluster of calcifications, or both, but these findings are not diagnostic of breast cancer. If mammographic studies are abnormal, either a breast biopsy is performed or the abnormality is reassessed with periodic follow-up mammograms until the nature of the abnormality is determined.(3) Unfortunately, mammography is far less sensitive and specific than the public perceives. Because of the lack of mammographic specificity, most biopsies of the breast are benign. In the US the probability of malignancy when a biopsy is performed on a mammographic abnormality usually ranges from 15-35%.(4-7) Consequently, for every woman who has a breast cancer detected by screening mammography, three to six women with false-positive results needlessly undergo the apprehension, pain and possible disfigurement associated with biopsy.

There is a pressing need for a widely available, non-invasive, and accurate imaging technique to further evaluate mammographically suspicious lesions to reduce the number of cancer negative biopsies. The need for a test with greater specificity than mammography also arises in patients with dense breast tissue, in patients with prior breast surgery, and in those who are at higher risk for breast cancer for whom the mammogram is equivocal.

Recent studies using radionuclides have suggested that scintimammography has high sensitivity and improves the specificity of conventional mammography for the detection of carcinoma of the breast and thus, deserves study as a means to reduce the number of mammographically "indicated" biopsies of the breast that yield negative results for carcinoma. For the agents having the best results to date (^{99m}Tc Sestamibi, ^{201}Tl, ^{99m}Tc MDP, and ^{18}F-FDG), tumor uptake is related to nonspecific factors such as metabolic rate, capillary permeability, and membrane ion transport. These small molecules also migrate into background tissues and reduce potential sensitivity by clouding the scintigraphic image. In contrast, ^{99m}Tc alpha-fetoprotein (^{99m}Tc AFP) most likely concentrates in breast cancer cells due to a specific receptor not found in normal adult breast tissue. In our models, ^{99m}Tc AFP has greater specificity for imaging breast cancer than other agents currently in clinical trial.

The work we are carrying out focuses on the development of ^{99m}Tc AFP as a novel agent to detect and stage breast cancer. We have studied both the utility of ^{99m}Tc AFP as an imaging agent and during the last year have worked to develop a radiopharmaceutical that can be widely available for clinical use in patients.

Purpose of Investigation

Our hypothesis is that AFP is concentrated in breast cancer cells by a receptor-mediated process, thereby providing a novel means for detecting breast cancer cells specifically and with high sensitivity. To test our hypothesis we pursued the following Specific Aims.

Aim 1. Identify the AFP molecule (natural full-length, recombinant full-length, recombinant domain III) that yields the highest tumor-to-background ratios in immune-deficient (SCID) mice bearing either ER+ MCF-7 or ER- MDA MB 231 human breast cancer xenografts.

Aim 2. Using the preparation of choice established in Aim 1, determine the breadth of applicability of ^{99m}Tc AFP as an imaging agent for human breast cancers. This was accomplished by comparing the imaging capability of ^{99m}Tc AFP to that of ^{201}Tl and ^{99m}Tc sestamibi in a broad range of different human tumor xenografts.

Aim 3. Measure the level of AFP receptors in the imaged tumors as a potential explanation for the imaging capability of this protein.

Aim 4. Determine whether ^{99m}Tc AFP can image metastases of a transplantable rat mammary cancer.

Scope of Work

Summary of effort on tasks abstracted from full work scope statement in original proposal:

Task 1: Months 1-4: AFP production.

Recombinant Domain III AFP: We produced Domain III of human AFP using a baculovirus vector which incorporates an N-terminal leader sequence from the baculoviral protein gp67, the Glutathione-S-Transferase protein from Schistosoma japonicium and Domain III of human AFP.

Natural human full length AFP: We purified human AFP secreted by HepG-2 cells using immunoaffinity chromatography, with purity and identity established by SDS-PAGE gels and Western Blots.

Task 2: Months 1-4: Grow human breast cancer cell lines as xenografts.

MCF-7 and MDA-MB-231 cell lines were expanded into multiple flasks, grown to confluence, harvested and centrifuged into cell pellets. Pellets are then solidified into fibrin clots, cut into pieces, and implanted into SCID mice.

Task 3: Months 3 & 4: Image MCF-7 and MDA-MB-231 breast cancer xenografts

Each of the AFP preparations was labeled with Tc-99m and injected i.v. into tumor xenograft-bearing mice. Mice were imaged and the ^{99m}Tc AFP preparations are assessed by their resultant tumor to background ratios (T/B) and clearance kinetics (% of injected dose/gram).

Task 4: Months 5-10: Establish AFP receptor assay using MCF-7 and MDA-MB-231 breast cancer cell lines

We proposed to radiolabel AFP with 125I by the Chloramin T method and reproduce the studies reported by Uriel(8). We then wished to evaluate the AFP receptor content of MCF-7 and MDA-MB-231. We were unable to achieve sufficient radiolabeled material to complete this task as originally proposed. An alternate procedure was developed to complete this task as described below.

Task 5: Months 5-24: Compare the imaging capability of ^{99m}Tc AFP to that of ^{99m}Tc sestamibi and ^{201} thallium

Each tumor line was expanded in culture and then transplanted into SCID mice. Replicate tumor-bearing mice are imaged in random order with ^{99m}Tc AFP, ^{99m}Tc sestamibi, and ^{201} thallium . Biodistribution kinetics and tumor to background ratios for each test agent was assessed. Studies were repeated for a variety of tumors.

Task 6: Months 5-24: Establish AFP receptor content in various tumors

We established AFP receptor content in various breast cancer tumor cell lines using a method designed to overcome the limitations encountered in task 4.

Task 7: Months 12-24: Lymphoscintigraphy studies of 99mTc AFP in rat homograft model of mammary adenocarcinoma metastasis to lymph nodes.

We obtained the 13762 NF rat mammary carcinoma cell line and transplanted it into the dorsal region of the upper neck in female Fischer rats. Because the animals died rapidly from the primary tumor mass, we were not able to establish the kinetics of metastasis to draining brachial and axillary nodes by necropsy and histopathology studies as was originally planned. Therefore, a model of pulmonary metastasis was established by direct injection of tumor cells i.v. Subsequently, ^{99m}Tc AFP was injected intravenously into tumor-bearing rats to image primary tumor and tumors established in the lungs of these animals.

Task 8: Months 25-36: (No cost extension year) Develop and test the imaging properties a 99mTc radiolabeled analogue of a minimal peptide sequence that retains the antiestrotrophic properties of full length AFP.

This task was not in the original proposal but became important to pursue given the success of the Albany Medical College AFP Research Group in identifying an AFP derived peptide that is the minimal sequence required to generate the antiestrotrophic activity of full length AFP. (See figure 6 in appendix) As with full length AFP and Domain III of AFP, this peptide exhibited dose-dependent inhibition of growth of estrogen-stimulated immature mouse uteri, the model used to predict therapeutic inhibition of estrogen dependent human tumors. Given the success of ^{99m}Tc radiolabeled full length and DIII in breast cancer imaging, we developed and tested procedures to radiolabel the peptide in a manner to preserve its biologic effectiveness in the mouse uterine assay. We then studied the imaging characteristics of the radiolabeled peptide and a control peptide in SCID mouse xenograft models of human breast and prostate cancer. The AFP peptide, like its larger relatives, shows specific localization in these tumors. Furthermore, necropsy studies suggest that the localization intensity is related to the amount of viable cells in the tumor mass.

BODY OF FINAL REPORT

Research Accomplishments by Task in Scope of Work

Task 1: Months 1-4: AFP production.

Recombinant Domain III AFP: We produced Domain III of human AFP using a baculovirus vector (pACSecG2T, Pharmingen) which incorporated an N-terminal leader sequence from the baculoviral protein gp67 (to facilitate secretion from insect cells), the Glutathione-S-Transferase (GST) protein from Schistosoma japonicium (to facilitate purification and solubilization of fusion protein) and Domain III of human AFP. The transfer vector containing the coding sequence for Domain III of hAFP was cotransfected into SF9 insect cells to produce recombinant virus. Virus was plaque-purified, then screened for the incorporation of the Domain III coding sequence into the viral genome (by PCR; Polymerase Chain Reaction) and for the ability of recombinant virus to produce secreted protein (Western Blot).

Recombinant baculovirus containing the cDNA for Domain III (previously described) was amplified by three serial passages and titered by plaque assay in order to produce a large quantity of virus. Protein was then produced in large batches by infection of SF9 cells with recombinant virus. The medium containing the secreted Domain III protein is harvested and clarified by centrifugation to remove cell debris which results from cell lysis. The protein is purified by loading the clarified cell culture medium onto a Glutathione-Agrose (Sigma) column. The column was washed with PBS and

then treated with Thrombin (Sigma) to release the Domain III fragment. The thrombin released fragment will was identified by Western Blot analysis using polyclonal antibody to human AFP and silver-stained SDS-PAGE. Protein was then aliquoted (50μg/each) and stored lyophilized at -80°C.

Natural human full length AFP: HepG2 cells were maintained and grown as a monolayer in αMEM (GIBCO, Grand Island, NY) supplemented with 5% serum (2/5 calf serum, 3/5 fetal calf serum), penicillin G (100 units/ml), and streptomycin (100 μg/ml). Cells were released from monolayer using 0.25% trypsin/0.25% EDTA. Subculturing into additional flasks was carried out by five-fold dilution of cells in the above maintenance medium. Confluent flasks were switched to serum-free medium to up-regulate production of AFP as described by Tecce et al. Serum-free medium is comprised of 3 parts αMEM: 1 part Waymouth's MB 752/1 plus 3 x 10^{-8} M sodium selenite, 2 mM L-glutamine and 1.5% antibiotic/antimycotic mixture from GIBCO. Cells were refed with serum-free medium every three days.

To purify AFP, HepG2 culture supernatants were pooled and concentrated using P-10 Centriprep concentrators (Amicon, Beverly, MA). Ten ml of concentrate containing approximately 3 mg of AFP were loaded onto an 18 cm x 2.5 cm immunoaffinity column (rabbit anti-human AFP (DAKO) conjugated to cyanogen bromide-activated Sepharose 4B) in a loading buffer of 100 mM NaCl/10 mM sodium phosphate pH 7.4. Concentrate was incubated on the column at room temperature for 30 minutes. Non-AFP proteins were eluted with approximately 200 ml loading buffer until no protein was detectable in the eluate by UV absorbance (280 nm). AFP is eluted with approximately 200 ml of 1.8 M $MgCl_2$ and dialyzed immediately against excess 10mM sodium phosphate buffer, pH 7.2. This material was washed and concentrated in a buffer comprised of 100 mM sodium chloride-10 mM sodium phosphate, pH 7.2.

This task has been completed for both recombinant Domain III AFP and natural human full length AFP.

Task 2: Months 1-4: Grow human breast cancer cell lines as xenografts.

MCF-7 and MDA-MB-231 cell lines were expanded into multiple flasks, grown to confluence, and harvested by trypsinization. Cells were converted to solid tumor form by centrifugation into a pellet and exposure of the cell pellet to 15 μl of fibrinogen (50 mg/ml) and 10 μl of thrombin (50 units/ml) for 30 minutes at 37°C. Fibrin clots were cut into pieces approximately 1.5 mm in diameter. Six to eight pieces were loaded into a 16 gauge trocar and were implanted subcutaneously in the region of the brachial lymph node near the front limb of female CB17 SCID mice. Tumors were usually palpable 3 to 4 weeks after implantation and reached a diameter of 1 cm approximately 6 weeks after implantation. To assess tracer sensitivity, imaging studies were performed when tumors were first palpable, approximately 0.2 cm in diameter, and as they enlarge to 1 cm in size.[9-11]

MCF-7 and MDA-MB-231 cell lines have been expanded, grown, harvested and implanted into SCID mice. The task is completed as proposed. In addition other cell lines have been implanted as xenografts as described in the results and discussion section.

Task 3: Months 3 & 4: Image MCF-7 and MDA-MB-231 breast cancer xenografts

^{201}Tl and ^{99m}Tc sestamibi are currently in clinical trial as imaging agents for breast cancer. Although early results have shown some promise [12-14], improvements in both sensitivity and specificity are needed. For example, a recent large multicenter trial enrolled 673 patients included 377 women with non-palpable mammographically detected abnormalities. The overall sensitivity and specificity for ^{99m}Tc sestamibi was 85% and 81% respectively with 72% sensitivity and 86% specificity for non-palpable tumors[15]. False positive sestamibi studies have been found in cases of fibrocys-

tic disease and in fibroadenomas. These false positive results may be due to hypercellularity and proliferative changes(16) or to the non-specific "metabolic activity" of these lesions.(13) Furthermore, ^{99m}Tc sestamibi is eliminated from the cell by the multidrug resistance P-glycoprotein (gp170) which is over expressed in some breast cancers.(17) Finally, ^{201}Tl and ^{99m}Tc sestamibi both produce images with variable patterns of normal breast activity that is in part due to significant scatter from non-specific uptake in cardiac and abdominal tissues.(18)

To evaluate the potential of ^{99m}Tc-AFP in imaging of breast cancer, each of the AFP preparations was labeled with Tc-99m and injected i.v. into tumor xenograft-bearing mice (see examples, Figure 1). Mice were imaged and the ^{99m}Tc AFP preparations were assessed by their resultant tumor to background ratios (T/B) and clearance kinetics (% of injected dose/gram).

^{99m}Tc labeling of AFP was carried out via stannous ion reduction as previously described (19). Briefly, Tc99m AFP was prepared from a 50μg AFP aliquot mixed with .5ml 0.9% Sodium Chloride

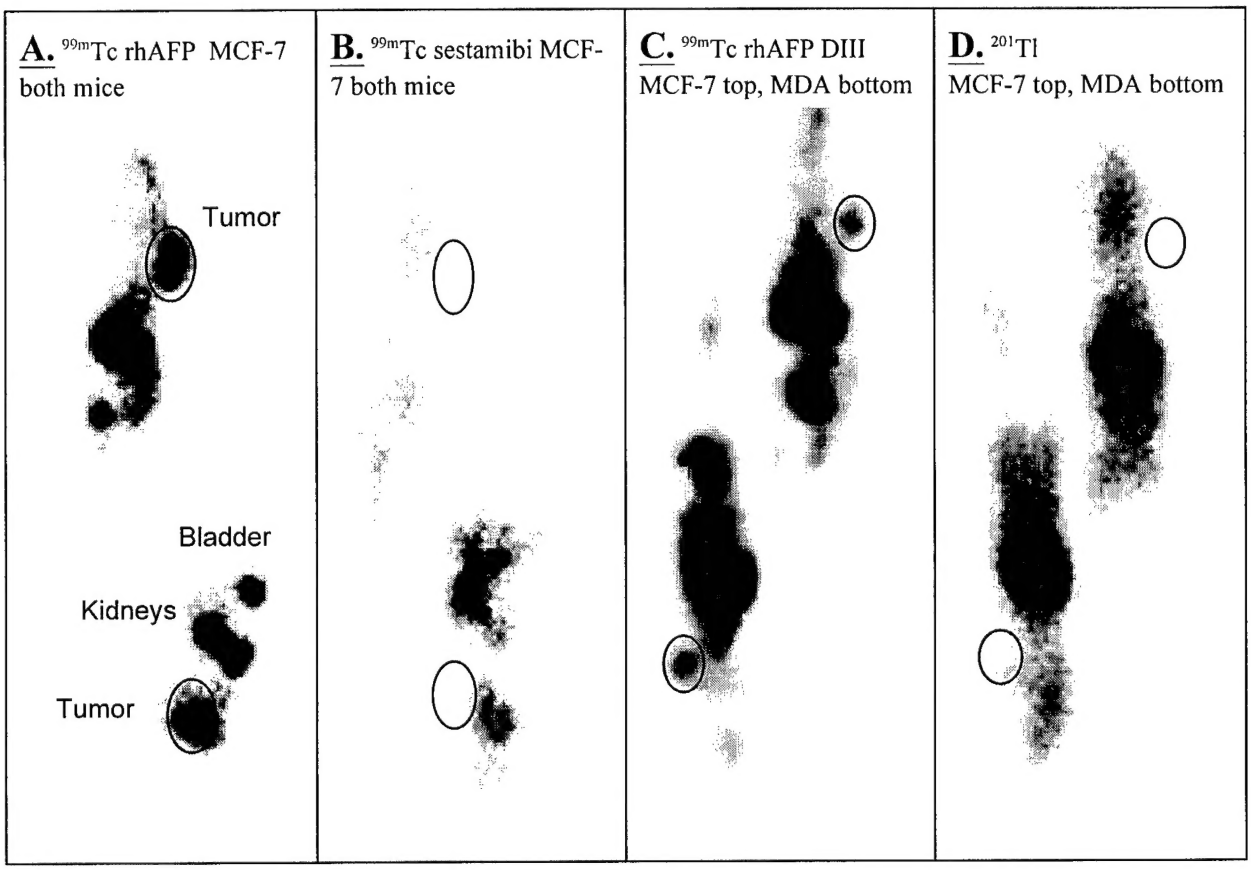

Figure 1: Sequential studies in mice bearing human tumor xenografts.

Panels A, B: 2 mice bearing MCF-7 ER+ human breast cancer xenografts 24 hours after doses of 37mbq of ^{99m}Tc AFP (A) and ^{99m}Tc sestamibi (B). Studies in panel A were performed 7 days after those in panel B in the same animals. Over this interval, tumors in right infra-axillary region were 1.2 cm in diameter. (about 900 mg)

Panels C, D: 2 mice bearing xenografts (top: MCF-7, bottom MDA MB-231 ER- tumor) 24 hours after doses of 37mbq of ^{99m}Tc AFP Domain III (C) and ^{201}Tl (D) Tumors in right infra-axillary region were 0.6cm in diameter (about 120 mg). Studies were 7 days apart (D after C) in the same animals.

Injection (Baxter Healthcare). The solution was added to an Ultra Tag RBC™ Reaction Vial (Mallinckrodt Medical Inc., St. Louis, MO), and the contents of the vial were mixed by gentle swirling, and incubated at room temperature for 5 minutes. At the completion of the incubation time, 20-1000 MBq ^{99m}Tc Sodium Pertechnetate Injection (Mallinckrodt Medical, Inc., St. Louis, MO) was added in a volume of 1-2 ml. The contents of the vial were mixed by gentle swirling and were incubated for 15 minutes. Dose aliquots were assayed using thin-layer chromatography performed on preparation using ITLC-SG (Gelman Instrument Co., Ann Arbor, MI) with acetone. Typically, 92 - 100% of the ^{99m}Tc was bound to AFP. The preparation was not used in studies if the percent bound was less than 90%.

In vivo biodistribution data was collected in up to 6 mice imaged simultaneously on a Siemens gamma camera. The data was collected by a dedicated computer and transferred to a Pentium PC MS Windows-based image processing system for analysis.[20,21] After sedation with intraperitoneal administration of 50 mg/kg pentobarbital, the mice were injected intravenously with 20-40 MBq of the tracer compound (^{99m}Tc AFP (3μg), ^{99m}Tc Sestamibi, or ^{201}Tl) and then were placed in the prone position on a thin polyethylene panel. To eliminate motion during imaging, the mice were restrained on panels by strips of tape over their extremities so as not to restrict respiration. Dynamic images obtained over 60 minutes were used to determine the biodistribution of the labeled agent. Typically, twelve sequential, five minute images were obtained with low energy general purpose collimation, and 1.5 hardware zoom into computer matrices having 128 by 128 picture elements.[20] Images were analyzed by positioning "regions of interest" (ROI) over the tumor, thigh, contralateral chest wall and total body. The counts were corrected for radioisotope decay to facilitate activity comparisons.

The image data were evaluated to determine the activity in the tumor tissue by drawing a region of interest that included all margins of the tumor. Where the tumors were not visibly apparent, regions were placed over the area where the tumor was palpated at the time of imaging. A second region was placed over the contralateral chest wall by reflecting the tumor region of interest through the

Breast Tumor Designation	**Histological and Biological Description**
MCF-7	ER+ breast cancers inhibited by Tamoxifen
T-47D	ER+ breast cancers inhibited by Tamoxifen
MDA MB 231	ER- breast cancers not inhibited by Tamoxifen
BT-20	ER- breast cancers not inhibited by Tamoxifen
Non-Breast Tumors	
MFE	Endometrial
MTW9A	Rat mammary tumor
LnCaP	AR+ prostate cancer
DU-145	AR- prostate cancer

Table 1: Cell lines tested. Cell lines included breast cancers and non-breast tumor, and non-tumor tissues. Each line was grown as a xenograft in SCID mice. The xenografts were used in studies to compare the tumor-imaging capability of ^{99m}Tc AFP to that of ^{99m}Tc Sestamibi and ^{201}Tl. (*ER = estrogen receptor, AR = androgen receptor)

midline axis. The second region was used to define a background activity that was subtracted from the tumor region to yield counts in the tumor tissue. The gram weight of the tumor was determined from a volume calculation based on measured tumor diameter. A third region of interest was defined over the thigh and the counts per gram of thigh tissue was obtained by using a thigh mass of 6% of body weight. A fourth region of interest was defined over the entire animal to determine the total injected activity. Regions were produced for each animal study at both 1 and 24 hour image collection points. Tracer localization parameters were determined by 1) the tumor uptake as the percent of injected activity per gram of tissue (%ID/gram); and 2) the tumor to background (TB) ratio determined from the ratio of tumor activity per gram to the thigh activity per gram.

Each of the AFP preparations (recombinant Domain III of human AFP and natural full length AFP) was labeled with Tc-99m and injected i.v. into tumor xenograft-bearing mice, completing the task as planned. Mice were imaged and the ^{99m}Tc AFP preparations were assessed by their resultant tumor to background ratios and clearance kinetics. Although we initially planned to focus on the best agent for further study (task 4), our initial results showed that both agents worked well and that there was not a clearly superior agent. We therefore proceeded to test both agents in xenografts of other tumors to characterize the relative performance of the agents. This task is complete (see attached manuscript).

Task 4: Months 5-24: Compare the imaging capability of ^{99m}Tc AFP to that of ^{99m}Tc sestamibi and ^{201} thallium

Each tumor line (Table 1) was expanded in culture and then transplanted into SCID mice. Replicate tumor-bearing mice are imaged in random order with ^{99m}Tc AFP, ^{99m}Tc sestamibi, and ^{201} thallium . Biodistribution kinetics and tumor to background ratios for each test agent is assessed. Studies are repeated for a variety of tumors.

The information from the preliminary studies we have carried out suggests that Tc-99m AFP will be an excellent imaging agent for human breast cancer. The studies of aim 1 were designed to allow us to compare the candidate Tc-99m AFP molecules in two human breast cancer xenografts (MCF7 an estrogen receptor positive (ER+) tumor and MDA MB 231, an ER- tumor cell line). The purpose of this aim was to identify which of the available ^{99m}Tc AFP molecules had the best tumor localization and imaging performance. This compound was to then be further tested in comparison with ^{99m}Tc Sestamibi and ^{201}Tl in a broad range of tumors. Image examples of the localization of the radiopharmaceuticals are shown in figure 2 of the attached manuscript. The quantitative comparisons of tumor percent injected dose/gram (%ID/gram) and tumor to background (T/B) are shown in Figure 2 and in Table 5 (see appendix). The results of the studies have been summarized using standard descriptive statistics. Comparison of differences between the different preparations have also been analyzed using analysis of variance with pair-wise comparisons done by Student-Newman-Keuls method.

We initially planned to compare ^{99m}Tc radiolabeled natural full length AFP, recombinant full length AFP and recombinant domain III of AFP (DIII). Unfortunately, the supply of recombinant full length AFP from Atlantic Biopharmaceuticals was interrupted by financial instability and subsequent failure of that corporation. Our studies with the two remaining forms of AFP showed better performance of full length natural AFP in the MCF7 xenografts whereas the DIII radiopharmaceutical appeared superior in the MDA MB 231 xenografts (see appendix). Given the reduced number of candidate AFP compounds and the uncertainty as to which of the remaining would prove to be superior, we decided to evaluate both the full length AFP and DIII in all of the tumors studied.

We evaluated the ^{99m}Tc AFP preparations by comparison to two other clinically available tracers that are being evaluated in patients with breast cancer: ^{99m}Tc sestamibi and ^{201}Tl. The tumors tissues

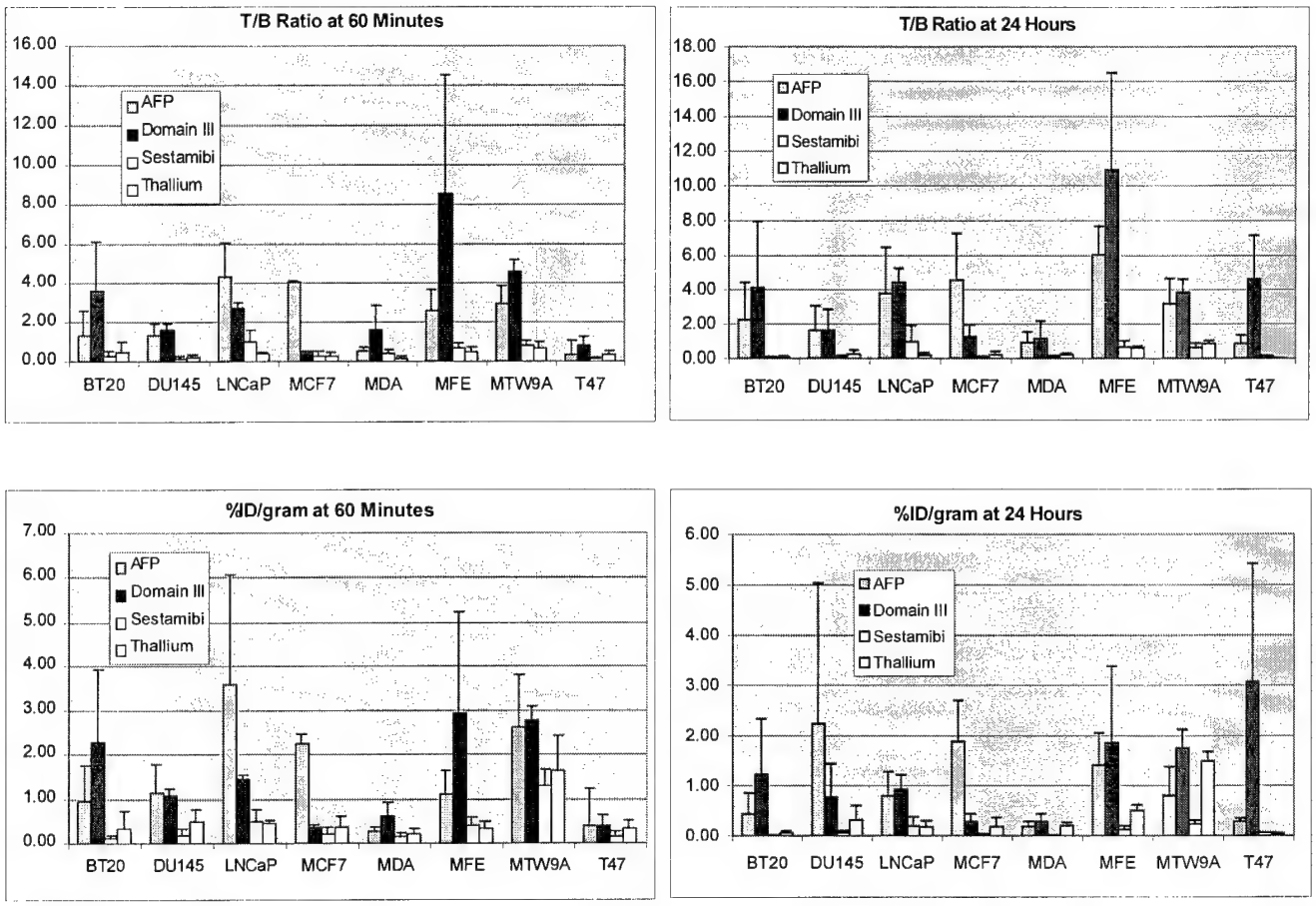

Figure 2: Mean and standard deviation of tumor measures at 1 and 24 hours.

represent breast and non-breast cancers that were steroid receptor positive or steroid receptor negative. Replicate tumor xenograft-bearing mice were used to assess the imaging capability of ^{99m}Tc AFP ^{99m}Tc sestamibi, and ^{201}Tl for each tumor. Four imaging studies were performed in a set of six replicate tumor xenograft-bearing mice, one for each tracer. The studies were spaced by 3-5 days to allow the previous tracer to decay and to allow the animals to fully re-equilibrate. The sequence of tracer administration was random. The tumor size and appearance was recorded over the interval to correlate size with detectability and target to background ratio.

The data were analyzed to address three specific questions. 1] Is radioactivity in tumor significantly above that in background tissue? 2] Is radioactivity in tumor from ^{99m}Tc AFP significantly above that from ^{99m}Tc sestamibi or ^{201}Tl? 3] Are there differences among tumors in radioactivity from ^{99m}Tc AFP?

We have completed the analysis of the data and have found that the results support the hypothesis that AFP is concentrated in breast cancer cells, thereby providing a novel means for detecting breast cancer cells specifically and with high sensitivity. Moreover, the results indicate that ^{99m}Tc AFP was a better imaging agent than ^{99m}Tc sestamibi and ^{201}thalium in all of the tumors studied (see appendix and manuscript-attached).

Task 5: Months 5-10: Establish AFP receptor assay using MCF-7 and MDA-MB-231 breast cancer cell lines

We proposed to radiolabel AFP with 125I by the Chloramin T method and reproduce the studies reported by Uriel[8]. We then wished to evaluate the AFP receptor content of MCF-7 and MDA-MB-231.

An assay for AFP receptor was developed by our group. The published procedure using radioiodinated AFP utilizes prohibitively large amounts of cold AFP[8]. Therefore, a technique employing only radioinert AFP was needed. We took advantage of the exquisite sensitivity of the Abbott IMχ immuno-quantitation of AFP (0.2 ng/ml) and the published report that AFP is dissociated from its receptor in 0.4 M KCl. 2.5 x 10^6 cells in 0.2 ml serum-free medium were incubated with varying concentrations of AFP for 3 hours at 4°C. Cells were washed four times by centrifugation and resuspension in serum-free medium. After the final washing there was no detectable AFP in the supernatant. Sodium azide (20 mM, 5 min, 4°C) was then added to prevent receptor-ligand complexes from internalizing when cells were subsequently warmed. KCl (0.4 M final concentration) was then added and incubated for one hour at 37°C. Cells were then centrifuged at 2,000 rpm for 10 minutes, supernatant is removed and AFP content in the supernatant was determined. By Scatchard plot analysis of AFP bound at different incubation concentrations, the number of specific binding sites per cell and their binding affinity were determined. Binding of AFP plateaus at 30 ng/ml. Bound AFP was approximately 0.1% of total AFP added to the cells. Therefore, concentration of free AFP was assumed to be equal to that of total AFP. The overall purpose of the task has been achieved albeit by a different route than originally planned. This task is therefore considered complete.

Task 6: Months 5-24: Establish AFP receptor content in various tumors

Using the methods developed in task 5, we carried out receptor binding studies of both breast and prostate cancers. The raw data yielded specific plus non-specific binding of probe to cells. Non-specific binding is estimated by performing identical incubations to these in the presence of a 50- to 100-fold excess of radioinert probe. The results of these manipulations are shown in Table 2.

The data indicate high-affinity binding of AFP to human cancer cells. This was true of prostate as well as breast cancer cells, regardless of whether breast cancer cells were positive or negative for estrogen receptor. The data are consistent with our imaging studies, which showed good uptake of AFP into all of the tumor xenografts that were evaluated. The data are also consistent with earlier studies, which have suggested that AFP receptor is expressed in dedifferentiated cells [8,22,23]. Re-

Table 2: AFP Receptor Binding Study Results

TUMOR (P:prostate, B: breast)	MDA **B / ER-**	MCF 7 B / ER+	T 47 B / ER+	LNCaP P / AR+
AFP Binding Kd	$6x10^{-8}$	$3.8 x10^{-8}$	$1.8x10^{-10}$	$3.9x10^{-9}$
Receptors - #/cell	140,000	43,800	30,200	31,000

lated studies by my collaborator, Dr. Bennett, have shown that AFP inhibits the growth of estrogen-receptor-positive, but not estrogen-receptor-negative, human breast cancer xenografts (Clin Cancer Res 4:2877-2884, 1998). Based on the imaging data and receptor data reported herein, the mechanism of this growth inhibition by AFP does not appear to be related to the uptake of AFP by these tumors.

Task 7: Months 12-24: Studies of 99mTc AFP in rat homograft model of metastasis.

To assess the ability of ^{99m}Tc AFP to detect spontaneous metastases we evaluated in the transplantable 13762 NF rat mammary adenocarcinoma model, which reliably metastasizes to draining lymph nodes within two weeks after transplantation into syngeneic rats (24).

We obtained the 13762 NF rat mammary carcinoma cell line and transplanted it into the dorsal region of the upper neck in female Fischer rats. The kinetics of metastasis to draining brachial and axillary nodes by necropsy and histopathology studies was investigated but scatter activity from the injection site made determination of activity in the lymphnode region unresolvable. We therefore developed a metastasis model system in which the investigation of metastasis uptake could be determined that did not require localized injection or would be confounded by uptake in the primary.

The 13762 NF rat mammary cancer cell line was obtained and grown as a monolayer in culture. Cells were harvested from culture and injected subcutaneously in rats. Tumors were palpable within 3-5 days following injection of 5 x 10^6 cells in the shoulder region of syngeneic Fischer rats.

Tumor became lethal 16-20 days after tumor implantation. Imaging was carried out when tumors were between 1.0 and 3.0 cm. ^{99m}Tc-AFP imaged this tumor quite well, yielding average tumor-to-background ratios of 3.9 and 4.3 at 1 and 22 hours, respectively. This compares, respectively, to a T/B of 5.4 and 4.7 for ^{99m}Tc -AFP in MCF-7 human breast cancer xenografts. Axillary node metastases were not detectable on necropsy until day 9 after tumor implantation, and lung metastases were not detectable until day 14 after tumor implantation. By these times the primary tumor was so large that it would have masked the imaging of spontaneous metastases. In order to circumvent this problem, an artificial model of lung metastases was established by injecting tumor cells intravenously. Tumor nodules were visible in the lung by day 9 after inoculation of 2 x 10^6 cells. Metastases are found only in the lung and are lethal by day 16 after implantation. As illustrated in figure 3, ^{99m}Tc-AFP has imaged lung nodules, with one area of the lung particularly intense on the imaging profile (circled). This area was hard on palpation during necropsy, and, on further examination, was found to contain a dense tumor nodule deep in the lung parenchyma.

Figure 3. 24 hour image of rat with a lung metastasis visible in apical region of right lung (circle). Smaller nodules are evident in the mid lung region (arrow)

TABLE 3. Activity of AFP-Derived Agents in Irnmature Mouse Uterine Growth Assay

Test Agent	Sequence	Dose*	Inhibition of Growth
Full-length Natural Albumin		100 ug	6
Full-length Natural Human AFP		100 ug	41**
Full-length Recombinant Human AFP		100 ug	34**
Recombinant Domain I ABC AFP		10 ug	0
Recombinant Domain III AB AFP		10 ug	32**
AFP Synthetic Peptide 447 (amino acids 447-480)		1 ug	36**
AFP Synthetic Peptide 457 (amino acids 457-466)		1 ug	0
Albumin Synthetic Peptide (amino acid 440-473)		1 ug	2
AFP Synthetic Peptide 467 (amino acid 467-480)	LCIRH EMTPV NPGV	1 ug	42**
AFP Synthetic Peptide 472 (amino acid 472-479)	EMTPV NPG	1 ug	49
AFP Synthetic Peptide 472 (amino acid 472-478)	EMTPV NP	1 ug	20
AFP Synthetic Peptide 473 (amino acid 473-479)	MTPV NPG	1 ug	0
AFP Tc-99m Chelator Peptide 472 with Tc label	GCGGQMTPV NPG	1 ug	40**
Chelator	GCGG	1 ug	0

*The optimal inhibitory dose per mouse given by the i.p. route. The optimal stimulatory dose of estrogen (0.5 Iag per mouse) was given by the i.p. route one hour after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to control groups which received no estrogen (negative control) or estrogen alone (positive control).

**Significant inhibition, $p<0.05$; Wilcoxon Sum of Rank Test

Task 8: Months 25-36 (no cost extension year): Studies of 99mTc AFP derived peptide.

In the last year of the project, a no cost extension year, we concentrated effort on the goal of defining a practical radiopharmaceutical compound that could be used in patient imaging studies. The concern that motivated this effort was the realization that the full length and DIII AFP molecules were either difficult to purify or produce in large quantity and would not likely be practical as a clinical radiopharmaceutical. The motivation for this effort was due, in part, to the success other members of the Albany Medical College AFP Research Group had in defining the minimum portion of the molecule that retained its growth regulation effect.

AFP is a glycoprotein produced during pregnancy by fetal yolk sac and by fetal liver and is a major protein constituent of fetal plasma throughout gestation [25]. The Albany AFP Research Group has been studying the regulation of breast cancer growth by AFP[9,26-29]. In those studies, full-length AFP, isolated from a variety of sources including a human hepatoma cell line (Hep G-2), stopped growth of estrogen-dependent (but not estrogen-independent) breast cancer growing as xenografts under the kidney capsule of immune deficient (SCID) mice. Part of the AFP Research Group's effort

has been directed toward finding the portion of the AFP molecule responsible for its inhibition of estrogen-stimulated cancer growth. Molecular biology tools were used to parse the AFP molecule into its domains and subdomains[29] and synthetic peptides from active subdomains were generated using solid phase peptide synthesis. These products were tested in a antiestrotrophic screening assay which measures inhibition of estrogen-stimulated growth of immature mouse uterus. As shown in Table 3, the antiestrotrophic activity is localized in a 8-mer peptide (amino acids 472-79).

The synthetic nature and defined structure of this octapeptide suggest that it can be developed into new drug that opposes the action of estrogen, perhaps including the promotional effects of estradiol in the development of human breast cancer. Further, preliminary data from our research group suggests that the octapeptide works by a mechanism other than that of tamoxifen.

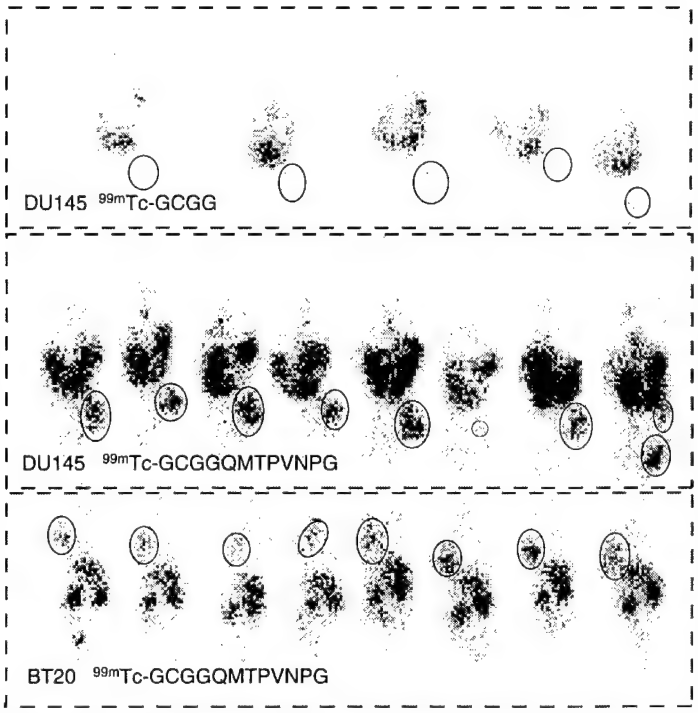

Figure 4. Comparison of in vivo targeting and biodistribution of 8-mer AFP derived peptide (with GCGG as chelator) within prostate (DU145) and breast (BT20) xenografts in SCID mice. Top row: Control study with tetrapeptide chelator GGCG in DU145. Middle row: AFP 8-mer peptide with GCGG chelator in DU145. Bottom row: AFP 8-mer peptide with GCGG chelator in BT20. Significantly higher localization is noted in the tumor (circles) compared to opposite shoulder. Accumulation of activity in the kidneys, liver and bladder is due to metabolism and excretion.

The data developed recently by the AFP group demonstrate that the octapeptide, P472-2 (amino acids 472-479 of AFP), possesses the entire antiestrotrophic activity contained in full-length AFP. Festin et al. [29] had previously shown that this activity was contained in the third domain of AFP (amino acids sequence 386-592), and Mizejewski et al[30] showed the activity resided in a 34-mer peptide (amino acid 447-480) from Domain III of AFP. The 8-mer peptide is a substantially truncated form of the 34-mer and yet retains significant activity. Smaller regions within this 8-mer were without activity. This AFP-derived octapeptide, if it behaves like full-length AFP, will be a novel agent in that it is not cytotoxic

	%ID/gram								Tumor	Tumor
	tumor	muscle	spleen	lung	heart	kidney	liver	blood	/blood	/muscle
Control DU145	0.22±0.06	0.07±0.02	0.68±0.29	0.50±0.12	0.19±0.05	6.77±1.89	2.98±0.66	0.19±0.04	1.16±0.18	3.33±0.62
AFP Peptide DU145	0.42±0.13	0.06±0.03	1.35±0.32	0.34±0.11	0.18±0.06	5.10±2.37	3.05±0.35	0.29±0.13	1.53±0.54	7.85±3.99
AFP Peptide BT20	0.43±0.05	0.03±0.01	1.03±0.08	0.27±0.04	0.11±0.02	2.28±0.48	2.84±0.25	0.18±0.03	2.34±0.08	12.76±2.31

Table 4: Necropsy study results in SCID mouse xenograft models of human breast (BT20) and prostate (DU145) cancer. Data shown as mean and standard deviation of necropsy tissue counts as percent of injected dose (%ID) per gram to correct for injected activity and sample weights. Control peptide was 99mTc GGCG, the chelator group used to bind 99mTc in the AFP peptide. Tumor to muscle ratios reflect imaging target to background and greater values imply better tumor detection capability in-vivo.

like most standard cancer chemotherapeutic agents. It is not an estrogen receptor antagonist like tamoxifen (unpublished data). It is highly likely that the peptide is anti-oncotic through a novel mechanism.

To evaluate whether the 8-mer would retain the imaging utility that the full length and DIII domains had shown, we developed a modified sequence that would allow direct chelation to Tc-99m through a tetramer with well established affinity for Tc-99m. After some methodologic development, we established a procedure to radiolabel the GCGGQMTPVNPG peptide with Tc-99m (Tc-99m AFPP) with high affinity (>95%). In brief, we produced ^{99m}Tc AFPP as purified by Sep-Pak C18 reverse phase chromatography after radiolabeling by ^{99m}Tc GHA transchelation. Our initial products were not stable after separation from the solvents used in reverse phase chromatography. We found that elution with 50%:50% ethanol:water and evaporation of the ethanol fraction under a stream of N2 produced a satisfactory product.

To evaluate the specific localization of the 99mTcAFPP we imaged and performed necropsy studies of SCID mice with BT20 breast cancer and DU145 prostate cancer human tumor xenografts

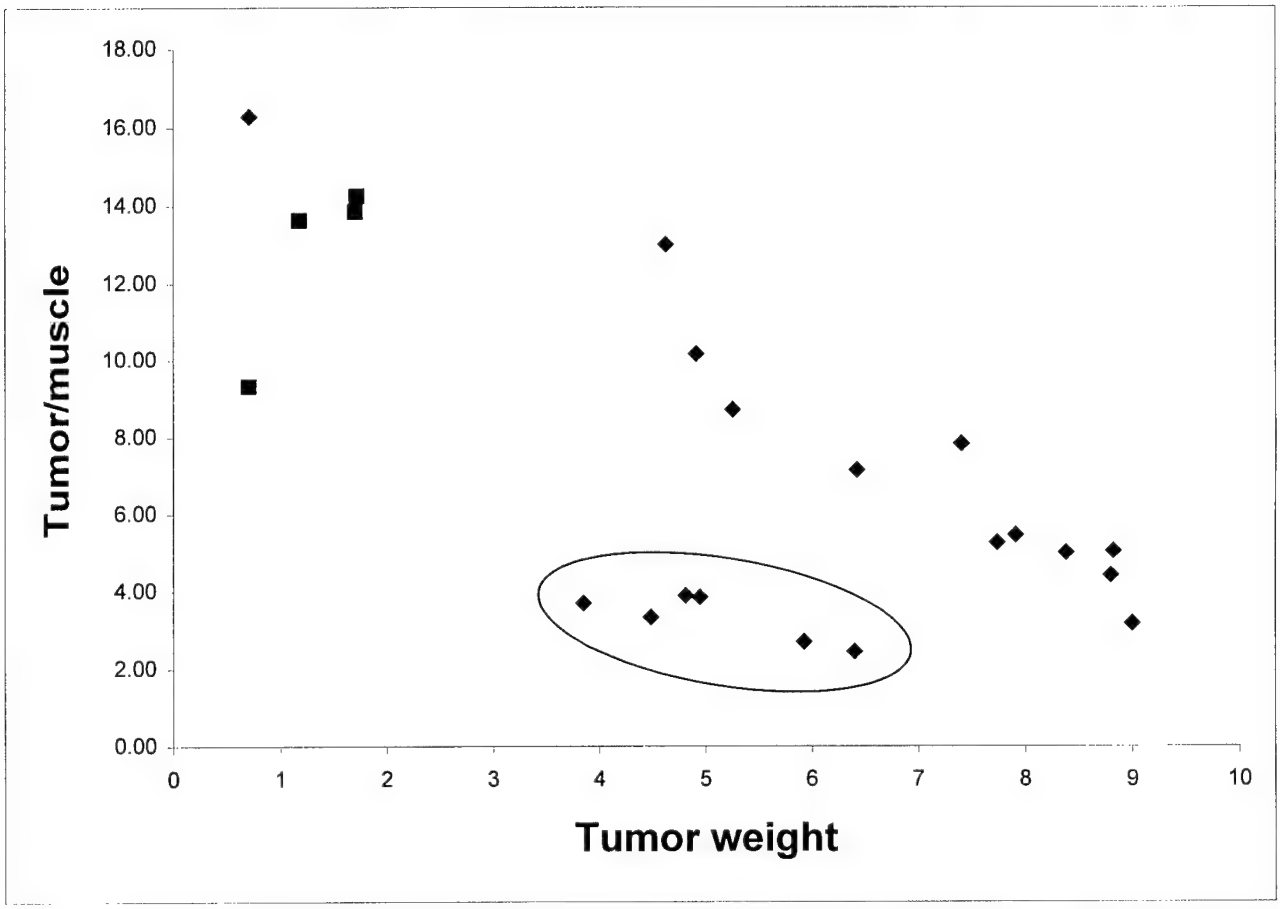

Figure 5. Tumor to muscle ratio as a function of tumor weight for 99mTc AFP peptide and 99mTc chelator control peptide in SCID mouse xenograft models of human breast and prostate cancer. The control peptide necropsy data (DU145) clusters with low tumor to muscle ratios (circled). 99mTc AFP peptide localized in BT20 xenografts (squares) and DU145 xenografts (diamonds) in a relatively linear relationship that suggests that uptake may be related to the amount of viable tissue in the tumorvolume. Tumors showed increasing quantities of necrosis with mass in these xenografts.

(Figure 4). Control animals (DU145 injected with the chelator peptide only) were compared to SCID mice bearing BT20 and DU145 xenografts necropsied 24 hours after injection (Table 4). The necropsy data suggests specific uptake of the AFP peptide in human breast and prostate tumor xenografts. At 24 hours post injection, tumor tissue localization is substantially higher than muscle, lung, heart, and blood. The spleen, kidney and liver are higher than tumor but not likely to interfere with diagnostic imaging detection of primary tumors and most breast and prostate metastases. The tumor to muscle ratios are substantially higher than control and as shown in figure 5 appear to be higher in smaller tumors, presumably because of the greater relative quantity of necrotic tissue in larger tumor volumes. A manuscript detailing these experiments is in preparation.

KEY RESEARCH ACCOMPLISHMENTS

Current methods of detecting breast cancer have low specificity and sensitivity. Although screening mammography results in early detection of breast cancer and reduces death from this disease, it has a low positive predictive value and a 60-90% false positive rate that leads to the pain, morbidity, and potential disfigurement associated with an estimated 500,000 unnecessary breast biopsies. Furthermore, true positive mammography is not helpful in assessing prognosis or predicting therapeutic response. Scintigraphic methods to detect breast cancer offer a means to improve the evaluation of patients with positive breast exams or positive mammograms.

Alpha-fetoprotein is a serum protein produced by fetal liver and crosses into the maternal circulation during pregnancy. Although developing tissues have the ability to bind and endocytose AFP, this function is lost by adult differentiated cells **(8,22,23)**. The capability reappears, however, in neoplastic cells growing either *in vivo* **(31)** or *in vitro* **(8,32,33)**. Malignant cells that have been shown to take up AFP include human breast cancer cells, malignant lymphoblastoid cells, neuroblastoma cells, and rhabdomyosarcoma cells **(8,34,35)**. Our collaborative "AFP group" has studied AFP and Domain III of human AFP because of their properties as inhibitors of breast cancer growth.**(11,36-39)** We have found that ^{99m}Tc radiolabeled recombinant human AFP (^{99m}Tc AFP) localizes rapidly and specifically in human breast cancer xenografts, providing well-defined images of the tumor relative to normal tissues.**(40)** Our studies indicate that ^{99m}Tc radiolabeled human AFP and domain III of human AFP show substantially better localization in human breast tumor xenografts than either ^{201}Tl or ^{99m}Tc sestamibi.**(40,41)** Furthermore, ^{99m}Tc AFP localizes in both estrogen receptor positive (ER+) MCF-7 and ER- MDA MB 231 breast tumors and has blood clearance through the kidneys. These features are critically important to an agent that must have high specificity.

Although scintimammography using non-specific tracers such as ^{99m}Tc sestamibi can improve the specificity of diagnostic imaging, it is likely that further gains in both specificity and sensitivity can be achieved with AFP because it is selectively taken up by breast cancer and has low non-specific uptake in normal tissues. The fact that our results indicate that ^{99m}Tc AFP demonstrates greater image specificity for breast cancer than either ^{201}Tl or ^{99m}Tc sestamibi suggests strongly that ^{99m}Tc AFP should be developed further for this purpose.

Tc-99m radiolabeled natural human AFP and the recombinant domain III of human AFP both have low non-specific tissue uptake and rapid renal clearance from blood. Localization of Tc-99m AFP in human breast cancer xenografts is initially rapid, increases with time, and is superior to Tc-99m sestamibi and Tl-201. Given its specific uptake by breast cancer cells, its low non-tumor localization and its rapid renal excretion, these Tc-99m AFP preparations are potential diagnostic agents for human breast carcinoma.

We have extended the potential utility of ^{99m}Tc AFP imaging by developing a peptide radiopharmaceutical that retains the pharmacologic and imaging characteristics of full length AFP but is more readily available and is likely to be superior as an imaging agent given its non-biologic source. This peptide derivative of the minimum sequence of AFP that retains the antiestrotrophic activity of AFP may prove to be of significant clinical utility in detecting human breast and prostate cancer.

REPORTABLE OUTCOMES

Bibliography of Publications and Meeting Abstracts

Line BR, Feustel P, Festin S, Andersen TT, Dansereau RN, Lukasiewicz RL, Zhu SJ, and Bennett JA. Scintigraphic Detection of Breast Cancer Xenografts with Tc-99m Natural and Recombinant Human Alpha-Fetoprotein. Cancer Biotherapy and Radiopharmaceuticals 1999; 14(6): 485-494.

Line BR, Bennett JA, Jacobson H, Andersen T, Feustel P, Dansereau RN. Scintigraphic Imaging of Human Breast and Prostate Cancer Xenografts with Tc-99m Labeled Alpha-Fetoprotein. J. Nucl. Med. 1998; 39:222P. Presented Society of Nuclear Medicine National Meeting 1998

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Mesfin FB, Ben ett JA, Jacobson HI, Zhu S, and Andersen TCT, Alpha-fetoprotein-derived antiestrotrophic ctapeptide, Biochimica et Biophysica Acta 1501(2000) 33-43

Funding based on outcomes of this award:

Evaluation of Radiolabeled Tumor Vessel Targeting Peptides as Novel Agents for the Staging and Therapy of Human Prostate Cancer PI: Bruce R, Line, M.D. 01/03/00-01/02/03 USAMRMC DAMD17-00-1-0004 Awarded 9/99 $567,853

Dextran Labeled Complex to Detect Tumor Angiogenesis PI: Bruce R, Line, M.D. 03/20/00 - 02/28/02 NIH 1R21 CA81492-01A1 Awarded 3/00 $312,000Published Abstracts of Work:

Line BR, Bennett JA, Jacobson H, Andersen T, Feustel P, Dansereau RN. Scintigraphic Imaging of Human Breast and Prostate Cancer Xenografts with Tc-99m Labeled Alpha-Fetoprotein. J. Nucl. Med. 1998; 39:222P.

Line BR, Dansereau RN, Andersen TT, Lukasiewicz RL, Bennett JA. Comparison of Tc-99m Human Alphafetoprotein to Tc-99m Sestamibi and Tl-201 in the Detection of Human Breast Cancer Xenografts. J. Nucl. Med. 1999; 40: 231P.

Developed Technique:

Semiquantitative image evaluation of tumor xenograft uptake based on tumor mass and soft tissue background. Reported in Line BR, et al. Scintigraphic Detection of Breast Cancer Xenografts with Tc-99m Natural and Recombinant Human Alpha-Fetoprotein. Cancer Biotherapy and Radiopharmaceuticals 1999; 14(6): 485-494

SUMMARY & CONCLUSIONS

Current methods of detecting breast and prostate cancer have low specificity and sensitivity. Receptors for AFP have been detected on many malignant cells but are not expressed by normal tissue cells. We studied the binding affinity of AFP for human breast and prostate cancer cell lines (Scatchard Analysis) and evaluated the imaging characteristics of Tc-99m full length AFP (AFP) and Tc-99m recombinant human AFP Domain III (DIII) relative to Tc-99m sestamibi and Tl201. Studies were carried out using human tumor cell lines from estrogen receptor (ER) positive (MCF7, T47) and ER negative (MDA-MB231) human breast cancers as well as androgen receptor (AR) positive (LNCaP) and AR negative (DU 145) prostate cancers. Tumor xenografts were placed in the lateral thorax region of CB-17 SCID mice and grown to a size of approximately 0.8-2.0 cm diameter (0.27-4 gm). Tracer kinetics were measured at 0-60 minutes and at 24 hours following injection of 37MBq of either Tc-99m AFP (4-6 μg), Tc-99m DIII (4-6 μg), Tc-99m sestamibi or Tl-201.

Our results suggest that breast and prostate tumors demonstrate high affinity receptors for AFP. Tc-99m AFP and Tc-99m DIII specifically localize in human breast and prostate cancers in vivo with higher target to background localization than either Tc-99m sestamibi or Tl-201. Tc-99m AFP and Tc-99m DIII show promise as imaging agents for the detection and staging of breast and prostate cancer. We have demonstrated similar improved imaging results with a radiolabeled peptide representing the minimum sequence of AFP that retains the full molecule antiestrotrophic activity. The peptide should provide a non-biologic, easily manufactured, agent that may realize the potential improvements in imaging of breast and prostate cancer obtained by Tc-99m AFP and Tc-99m DIII.

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APPENDICES

Structure of AFP and Subdomains

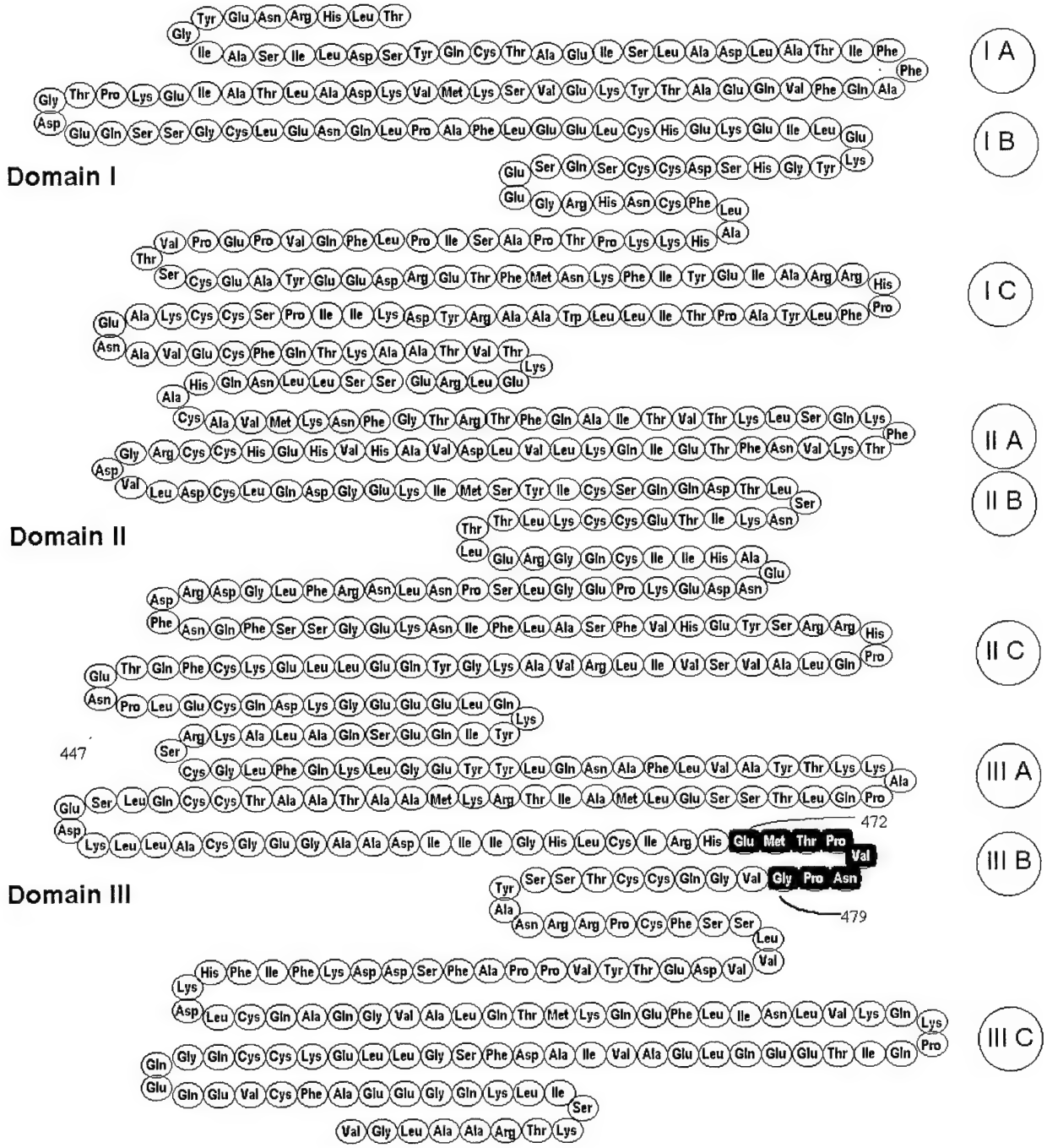

Figure 6. Amino acid sequence of full length human AFP. The domain III sequence from amino acid 447 contains the minimum sequence #472-479 required to generate the anti-estrotrophic activity of full length AFP.

Tables of Supporting Data

Table 3A: Percent Injected Dose per Gram Tumor at 1 hour

Tumor	AFP	Domain III	Sestamibi	Thallium
BT20	[8] 0.98 (0.80)	[4] 2.30 (1.62)	[7] 0.12 (0.08)	[7] 0.33 (0.40)
DU145	[8] 1.16 (0.64)	[3] 1.08 (0.15)	[5] 0.18 (0.16)	[4] 0.48 (0.29)
LNCaP	[10] 3.60 (2.48)	[5] 1.46 (0.09)	[5] 0.49 (0.29)	[4] 0.45 (0.08)
MCF7	[2] 2.25 (0.22)	[2] 0.38 (0.06)	[5] 0.22 (0.14)	[6] 0.36 (0.27)
MDA	[6] 0.27 (0.09)	[10] 0.62 (0.31)	[6] 0.17 (0.09)	[6] 0.21 (0.15)
MFE	[5] 1.11 (0.54)	[5] 2.96 (2.28)	[4] 0.39 (0.19)	[6] 0.34 (0.16)
MTW9A	[5] 2.64 (1.16)	[4] 2.79 (0.30)	[3] 1.31 (0.37)	[5] 1.63 (0.82)
T47	[2] 0.39 (0.85)	[8] 0.39 (0.25)	[2] 0.14 (0.13)	[3] 0.34 (0.18)

Table 3B: Percent Injected Dose per Gram Tumor 24 hrs

Tumor	AFP	Domain III	Sestamibi	Thallium
BT20	[8] 0.44 (0.43)	[4] 1.23 (1.11)	[7] 0.01 (0.01)	[7] 0.06 (0.04)
DU145	[8] 2.25 (2.80)	[3] 0.77 (0.67)	[5] 0.06 (0.04)	[4] 0.32 (0.29)
LNCaP	[7] 0.80 (0.48)	[5] 0.93 (0.30)	[5] 0.20 (0.19)	[4] 0.17 (0.13)
MCF7	[2] 2.00 (1.89)	[2] 0.29 (0.16)	[2] 0.03 (0.01)	[5] 0.18 (0.18)
MDA	[6] 0.19 (0.10)	[12] 0.28 (0.16)	[6] 0.01 (0.01)	[6] 0.21 (0.06)
MFE	[5] 1.42 (0.64)	[5] 1.86 (1.52)	[4] 0.12 (0.08)	[6] 0.51 (0.11)
MTW9A	[5] 0.81 (0.58)	[4] 1.75 (0.38)	[3] 0.24 (0.08)	[4] 1.49 (0.19)
T47	[2] 0.29 (0.06)	[8] 3.07 (2.35)	[2] 0.07 (0.00)	[3] 0.04 (0.03)

Table 3C: Tumor to Thigh Tissue Background Ratio 1 hour

Tumor	AFP	Domain III	Sestamibi	Thallium
BT20	[8] 1.30 (1.28)	[4] 3.59 (2.54)	[7] 0.28 (0.26)	[7] 0.46 (0.53)
DU145	[8] 1.33 (0.58)	[3] 3.00 (1.60)	[5] 0.13 (0.12)	[4] 0.22 (0.13)
LNCaP	[10] 4.32 (1.77)	[5] 2.73 (0.28)	[5] 0.99 (0.61)	[4] 0.38 (0.05)
MCF7	[2] 4.10 (0.01)	[2] 0.55 (0.01)	[5] 0.29 (0.23)	[6] 0.28 (0.22)
MDA	[6] 0.56 (0.20)	[10] 1.57 (1.31)	[6] 0.41 (0.20)	[6] 0.15 (0.11)
MFE	[5] 2.60 (1.09)	[5] 8.50 (6.02)	[4] 0.65 (0.30)	[6] 0.49 (0.22)
MTW9A	[5] 2.95 (0.94)	[4] 4.62 (0.60)	[3] 0.81 (0.22)	[5] 0.65 (0.33)
T47	[2] 0.30 (0.78)	[8] 0.78 (0.47)	[2] 0.11 (0.09)	[3] 0.34 (0.21)

Table 3D: Tumor to Thigh Tissue Background Ratio 24 hrs

Tumor	AFP	Domain III	Sestamibi	Thallium
BT20	[8] 2.26 (2.18)	[4] 4.13 (3.85)	[7] 0.05 (0.07)	[7] 0.09 (0.06)
DU145	[8] 1.65 (1.43)	[3] 1.65 (1.22)	[5] 0.10 (0.06)	[4] 0.26 (0.24)
LNCaP	[7] 3.79 (2.70)	[5] 4.44 (0.82)	[5] 0.97 (0.95)	[4] 0.19 (0.13)
MCF7	[2] 4.56 (2.71)	[2] 1.28 (0.68)	[2] 0.09 (0.04)	[5] 0.20 (0.20)
MDA	[6] 0.93 (0.61)	[12] 1.17 (1.00)	[6] 0.10 (0.08)	[6] 0.23 (0.08)
MFE	[5] 6.04 (1.66)	[5] 10.93 (5.58)	[4] 0.65 (0.39)	[6] 0.61 (0.11)
MTW9A	[5] 3.21 (1.45)	[4] 3.87 (0.75)	[3] 0.63 (0.26)	[4] 0.87 (0.17)
T47	[2] 0.86 (0.49)	[8] 4.62 (2.54)	[2] 0.15 (0.01)	[3] 0.03 (0.02)

Data shown as number of animals in brackets, mean and standard deviation in parentheses (see description in body of report).

Bibliography of Publications and Meeting Abstracts

Line BR, Feustel P, Festin S, Andersen TT, Dansereau RN, Lukasiewicz RL, Zhu SJ, and Bennett JA. Scintigraphic Detection of Breast Cancer Xenografts with Tc-99m Natural and Recombinant Human Alpha-Fetoprotein. Cancer Biotherapy and Radiopharmaceuticals 1999; 14(6): 485-494.

Line BR, Bennett JA, Jacobson H, Andersen T, Feustel P, Dansereau RN. Scintigraphic Imaging of Human Breast and Prostate Cancer Xenografts with Tc-99m Labeled Alpha-Fetoprotein. J. Nucl. Med. 1998; 39:222P. Presented Society of Nuclear Medicine National Meeting 1998

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List of Personnel

Dr. Bruce R. Line a board certified Nuclear Medicine Physician who has specialized in clinical trials and the basic science of radiopharmaceutical development and image analysis. He coordinated the work of the research assistants and interacted with his co-investigators on a weekly basis. Dr. Line will managed study protocols, imaging and analysis, and interacted with the radiopharmacist and statistical staff. He was responsible for grant related duties and project publications.

Dr. James A. Bennett is a Cancer Pharmacologist who has worked in the area of experimental therapeutics of cancer for 20 years. He was responsible for supervising the research assistants in cell culture preparations and the rodent model xenograft and homograft that were utilized in the proposal. He was responsible for providing the hepatoma derived AFP. Dr. Bennett assisted Dr. Line in data evaluation, result presentation, progress reports and manuscript authorship. Dr. Bennett has worked closely with the other investigators in the "AFP Group" at Albany Medical College for a number of years, and his expertise was an important contribution to the environment in which the work will be performed.

Dr. Thomas T. Andersen is a physical biochemist with extensive experience in protein chemistry, molecular biology, and endocrinology. An Associate Professor in the Department of Biochemistry & Molecular Biology, and Director of the Protein Chemistry Core Facility, Dr. Andersen's laboratory produced, purified and analyzed the recombinant human AFP preparations for the studies. Dr. Andersen assisted Dr. Line in data evaluation. He is a member of the "AFP Group" at Albany Medical College.

Paul J. Feustel, Ph.D. is a Professor in the Departments of Surgery and Physiology/Cell Biology. He serves as a statistical consultant for investigators and students in Albany Medical College and at Rensselaer Polytechnic Institute. He is proficient in the use of software (BMDP, SAS, SOLO, MathCAD) for statistical analysis, power analysis for experimental design, and mathematical modeling.

Herbert I Jacobson, Ph.D. is a Professor in the Department of Obstetrics and Gynecology. He has been a central member of the AFP group and was responsible for assessment of the binding of AFP to cells and studies of specific versus non-specific uptake.

Shuji Zhu, M.D. is a full-time research assistant who has worked on the project for the past five years. A retired general surgeon from mainland China, she carried out the cell culture techniques and with the xenograft implantation techniques required for this project.

Roberta L Lukasiewicz is a full-time research assistant necessary for support of the animal imaging studies, necropsies, gamma counting, image processing and data analysis. She worked full time in Dr. Line's laboratory on the project preparations and analytical procedures. She assisted Dr. Line in several facets of the animal preparation, imaging studies and data reduction.

CANCER BIOTHERAPY & RADIOPHARMACEUTICALS
Volume 14, Number 6, 1999
Mary Ann Liebert, Inc.

Scintigraphic Detection of Breast Cancer Xenografts with Tc-99m Natural and Recombinant Human Alpha-Fetoprotein

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Because adenocarcinoma of the breast expresses receptors for alpha-fetoprotein (AFP), we studied Tc-99m AFP as a radiopharmaceutical to detect breast cancer. The biodistribution of Tc-99m radiolabeled natural human AFP (full length) and recombinant domain III (DIII) of human AFP was compared to Tc-99m sestamibi and Tl-201 in a murine model of human breast cancer. Estrogen receptor positive (MCF7, T-47D) and estrogen receptor negative (MDA-MB-231, BT-20) human breast cancer xenografts were grown subcutaneously in the lateral thorax region of immunosuppressed mice (ICR SCID). Quantitative comparisons of percent-injected dose per gram of tissue (%ID/gram) and tumor to thigh ratio (T/Th) were performed at 0-60 minutes and at 24 hours following injection. For most tumors, T/Th for AFP and DIII was significantly greater than T/Th for Tc-99m sestamibi and Tl-201. In all breast cancers (BT-20, MCF7, MDA-MB-231, T-47D), Tc-99m AFP T/Th increased from 60 minutes to 24 hours, suggesting good tumor retention of this radiopharmaceutical. DIII and AFP had significantly higher %ID/gram than either Tl-201 or Tc-99m sestamibi when considered across all tumor types at both 60 minutes and 24 hours. The data suggests that localization of Tc-99m AFP in human breast cancer xenografts is initially rapid, increases with time, and is superior to Tc-99m sestamibi and Tl-201. Given its high uptake by breast cancer cells, its low non-tumor localization and its rapid renal excretion, these Tc-99m AFP preparations may be useful agents to detect human breast carcinoma.

Key words: Alpha-fetoprotein, scintigraphic imaging, technetium-99m radiopharmaceutical, human tumor xenografts

INTRODUCTION

Current methods of detecting breast cancer have low specificity and sensitivity.[1-3] Because of the lack of mammographic specificity, most biopsies of the breast are benign, with the probability of malignancy ranging from 15-35%.[4-7] The need for a test with greater specificity than mammography also arises in patients with dense breast tissue, in patients with prior breast surgery, and in those who are at higher risk for breast cancer for whom the mammogram is equivocal. Scintimammography is a widely available, non-invasive, and useful imaging technique that is now being explored as a method to further evaluate mammographically suspicious lesions and improve

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the specificity of breast cancer detection. However, the potential of scintimammography is determined by the characteristics of the radiopharmaceutical used in the study.

Alpha-fetoprotein (AFP) is produced by the fetal liver and is present in fetal and maternal circulation during pregnancy. The gene for this protein is repressed at parturition. Certain dedifferentiated tissues actively take up AFP, presumably as part of a fatty acid transport system.[8-11] It is also taken up by certain cancer cell lines in vitro, including human breast cancer.[12-14] The affinity of AFP for human breast cancer cells in vitro and the lack of receptors in differentiated tissue suggest that a radiolabeled molecule of AFP may be useful as a means to detect malignancy, particularly breast cancer. Therefore, we studied the imaging potential of Tc-99m radiolabeled human AFP and its recombinant domain III (DIII) fragment in xenograft models of human breast cancer in immune deficient mice and compared image data obtained with these agents to that obtained with Tc-99m sestamibi and Tl-201.

MATERIALS AND METHODS

Preparation of Natural Human Full Length AFP From Human Hepatoma (Hepg2) Cells

HepG2 cells were maintained and grown as a monolayer in αMEM (GIBCO, Grand Island, NY) supplemented with 5% serum (2/5 calf serum, 3/5 fetal calf serum), penicillin G (100 units/ml), and streptomycin (100 μg/ml). Cells were released from monolayer using 0.25% trypsin/0.25% EDTA. Subculturing into additional flasks was carried out by five-fold dilution of cells in the above maintenance medium. Confluent flasks were switched to serum-free medium to up-regulate production of AFP as described by Tecce et al.[15] Serum-free medium was comprised of 3 parts αMEM: 1 part Waymouth's MB 752/1 plus 3 x 10^{-8} M sodium selenite, 2 mM L-glutamine and 1.5% antibiotic/antimycotic mixture (GIBCO, Grand Island, NY). Cells were refed with serum-free medium every three days.

HepG2 culture supernatants were pooled and concentrated using P-10 Centriprep concentrators (Amicon, Beverly, MA). AFP was purified from the supernatant by loading 10 ml of concentrate containing approximately 3 mg of AFP onto an 18 cm x 2.5 cm immunoaffinity column (rabbit anti-human AFP conjugated to cyanogen bromide-activated Sepharose 4B) in a loading buffer of 100 mM sodium chloride/10 mM sodium phosphate pH 7.4. Concentrate was incubated on the column at room temperature for 30 minutes. Non-AFP proteins were eluted with approximately 200 ml loading buffer until no protein was detectable in the eluate by UV absorbence (280 nm). AFP was eluted with approximately 200 ml of 1.8 M $MgCl_2$ and dialyzed immediately against excess 10 mM sodium phosphate buffer, pH 7.4. This material was washed and concentrated in a buffer comprised of 100 mM sodium chloride/10 mM sodium phosphate, pH 7.4. The purified protein yielded a single band on silver-stained gels, and the band was identified as AFP by Western blot using polyclonal antibody to human full length AFP (Dako Corp., Carpinteria, CA).

Recombinant Domain III AFP

The third domain of AFP (DIII) was produced using a baculovirus expression system. DIII was chosen because previous studies had shown that the biological activity of AFP was localized to the third domain of the molecule.[16,17] The cDNA encoding all of DIII and a small part of domain II was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cDNA was amplified by polymerase chain reaction (PCR) to yield a fragment that encoded for DIII only. This fragment was subcloned in a cloning vector (TOPO™ TA Cloning® Kit, Invitrogen, Inc, Carlsbad CA) in order to provide convenient overlapping ends (5'*Xho* I site and 3'*Nsi* I site). The subcloning step permitted directional cloning of the cDNA into a transfer vector (5'*Xho* I site and 3' *Nsi* I compatible with *Pst* I) downstream of a polyhedron promoter and flanked by viral sequences necessary for allelic replacement. The transfer vector (pAcSGHisNT-B, Pharmingen, Inc., Franklin Lakes, NJ) was modified to include a segment that codes for a polyHistidine region on the N-terminus of each DIII AFP to facilitate purification by metal ion affinity chromatography. PAcSGHisNT-DIII was inserted into modified Autographica californica nuclear polyhedrosis virus to generate a plasmid capable of producing a histidine-tagged DIII fusion protein. Plasmid was

grown in Sf9 cells (Invitrogen) and transferred to High Five cells (Invitrogen) to obtain amplified protein expression of DIII AFP (including the polyHistidine tag at the N-terminus). After the protein was purified on an affinity column designed to bind the His linker (Talon resin, Clonetech, Inc., Palo Alto, CA) and was eluted with imidazole, it yielded a single band on silver-stained gels. This band was identified as AFP by Western blot using polyclonal antibody to human full-length AFP (Dako Corp., Carpinteria, CA). Protein identity and quantity were further verified by amino acid analysis.

Human Breast Cancer Cell Lines

The MCF7 and MDA-MB-231 human breast cancer cell lines were obtained from ATCC, and were grown in DMEM supplemented with 5% fetal calf serum, 1% non-essential amino acids, 10 ng/ml insulin, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. The BT-20 human breast cancer cell line (ATCC) was grown in EMEM supplemented with 10% fetal calf serum, 1% non-essential amino acids and penicillin/streptomycin as above. The T-47D human breast cancer cell line (ATCC) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 µg/ml insulin, L-glutamine and penicillin/streptomycin as described above. MCF-7 and T-47D are estrogen receptor positive breast cancers and grow more slowly as xenografts that the estrogen receptor negative MDA-MB-231 and BT-20 breast cancers. The MFE-296 (ATCC) is a human endometrial carcinoma and was grown in EMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 1 µg/ml insulin, 2.5 µg/ml transferrin and penicillin/streptomycin as above. The MTW9A (gift from Untae Kim, Roswell Park Cancer Institute, Buffalo, NY) is an estrogen receptor positive rat mammary cancer. It was maintained by serial transplantation in Wistar Furth female rats (Harlan, Indianapolis, IN) and then transplanted as a xenograft in ICR-SCID mice.

Human Breast Cancer Xenografts

ICR severe combined immunodeficiency (SCID) female, five-week-old mice (Taconic Farms, Germantown, NY) were housed in filter-covered cages under laminar flow conditions and fed autoclaved mouse chow and water ad libitum. Human tumor pieces for transplantation into mice were obtained from cell lines that were released from monolayer by trypsinization, diluted into single-cell suspension and then solidified by centrifugation into a pellet. The pellet was encased in clot by exposure to 15 µl fibrinogen (50 mg/ml) (Sigma, St. Louis, MO) and 10 µl thrombin (50 units/ml) (Armour Pharmaceutical Company, Kankakee, IL) for 30 minutes at 37°C.[18] The fibrin clots containing tumor were cut into pieces approximately 1.5 mm in diameter. Six to eight pieces were loaded into a 16-gauge trocar and implanted subcutaneously in the lateral thoracic region of mice. Estrogen supplementation of mice was achieved by subcutaneous implantation of a Silastic tubing capsule containing solid estradiol (Sigma, St. Louis, MO) on the day of tumor implantation. Tumor growth was monitored by weekly measurement of tumor size using a vernier caliper. Tumors usually became palpable 3 weeks after implantation and attained a size of 1 cm in diameter 5 weeks after implantation.[18-20] To assess tracer sensitivity, imaging studies were performed when tumors were approximately 0.5 cm in diameter, and as they enlarged to 1.5 cm in size.

Radiolabeling Procedure

Tc-99m labeling of AFP (full length and DIII) was carried out via stannous ion reduction as previously described.[21] Briefly, Tc99m AFP was prepared from a 50 µg AFP aliquot mixed with 0.5ml 0.9% Sodium Chloride Injection (Baxter Healthcare Deerfield, IL). The solution was added to an Ultra Tag RBC™ Reaction Vial (Mallinckrodt Medical Inc., St. Louis, MO), and the contents of the vial were mixed by gentle swirling, and incubated at room temperature for 5 minutes. At the completion of the incubation time, 370-740 MBq Tc-99m Sodium Pertechnetate Injection obtained from a freshly eluted generator (Mallinckrodt Medical, Inc., St. Louis, MO) was added in a volume of 1-2 ml. The contents of the vial were mixed by gentle swirling and were incubated for 15 minutes at room temperature. Dose aliquots were assayed for integrity of binding of Tc-99m to AFP using thin-layer chromatography. Typically, 92 - 100% of the Tc-99m was bound to AFP. The preparation was not used in studies if the percent bound was less than 90%. To define the

stability of the Tc-99m AFP preparations, thin-layer chromatography in acetone (ITLC-SG, Gelman Instrument Co., Ann Arbor, MI) was again used to determine the degree of Tc-99m that was unbound with shelf storage for 3 and 6 hours at room temperature. Sestamibi was radiolabeled using kits for the preparation of Tc-99m Sestamibi Injection (DuPont Pharma, Inc., N. Billerica, MA).

Tracer Biodistribution and Imaging Studies

In vivo biodistribution data was collected in up to 6 mice imaged simultaneously on a gamma camera. After sedation with intraperitoneal administration of 50 mg/kg pentobarbital, the mice were injected intravenously with 20-40 MBq of the tracer compound (Tc-99m AFP (3μg), Tc-99m sestamibi, or Tl-201) and then were placed in the prone position on the gamma camera collimator. To eliminate motion during imaging, the mice were restrained by strips of tape over their extremities so as not to restrict respiration. Twelve sequential, five minute images were obtained with low energy general purpose collimation, and 1.5 fold hardware zoom into computer matrices having 128 by 128 picture elements.[22] The animals were reanesthetized at 24 hours post injection and two thirty minute static images were obtained. The data was transferred to a Pentium PC MS Windows-based image processing system for analysis.[22,23]

The image data were evaluated to determine the activity in the tumor tissue by drawing a specific region of interest that included all margins of the tumor. Where the tumors were not scintigraphically visible, regions were placed over the area where the tumor was palpated at the time of imaging. A second region was placed over the contralateral chest wall by reflecting the tumor region of interest through the midline axis. The second region was used to define a background activity that was subtracted from the tumor region to yield counts in the tumor tissue. The gram weight of the tumor was determined from the tumor volume, assuming spheroid geometry, as calculated from the measured tumor diameter. A third region of interest was defined over the thigh and the counts per gram of thigh tissue was obtained by using a thigh mass of 6% of body weight. A fourth region of interest was defined over the entire animal to determine the total injected activity. Regions were produced for each animal study at both 60 minute and 1440 minute (24 hr) image collection points. Tracer localization parameters were determined by 1) the tumor uptake as the percent of injected activity per gram of tissue (%ID/gram); and 2) the tumor to thigh tissue background (T/Th) ratio determined from the ratio of tumor activity per gram to the thigh activity per gram.

Statistical Analysis Methodology

For each tumor, all four tracers were studied in the same six tumor bearing mice with a one-week rest period between the administration of each tracer. The order in which the tracers were injected for each tumor type was randomly chosen. Standard descriptive statistics (mean and standard deviation) were used to summarize the percent-injected dose/gram of tumor (%ID/gram) and tumor to thigh ratio (T/Th) results. Analysis of %ID/gram and T/Th ratios was by repeated measures of analysis of variance (ANOVA) with fixed main effects of tumor type and tracer. Repeated measures of analysis included a main effect of time (for the 60 and 1440-minute determinations) as well as interactions (tracer with tumor, tracer with time, tumor with time and tumor with tracer with time). When justified by statistically significant main or interaction effects, multiple comparisons were performed by the Student Newman Keuls multiple range test.

RESULTS

In-Vivo Studies of Tc-99m AFP Uptake by Human Breast Cancer Xenografts

Using both Tc-99m radiolabeled preparations of full length human AFP and its recombinant DIII, SCID mice bearing either estrogen receptor positive (MCF7, T-47D, MTW9A) or estrogen receptor negative (MDA-MB-231, BT-20) breast cancer xenografts were imaged continuously over one hour and then re-imaged at 24 hours. The mice showed little accumulation of tracer by any normal tissue other than kidney during the first hour after injection. By 24 hours, the tracer was cleared from the blood and normal tissues, and was found primarily in the kidneys and bladder (Figure 1).

We compared the imaging capability of the Tc-99m AFP preparations to two other clinically available tracers that are being evaluated in patients

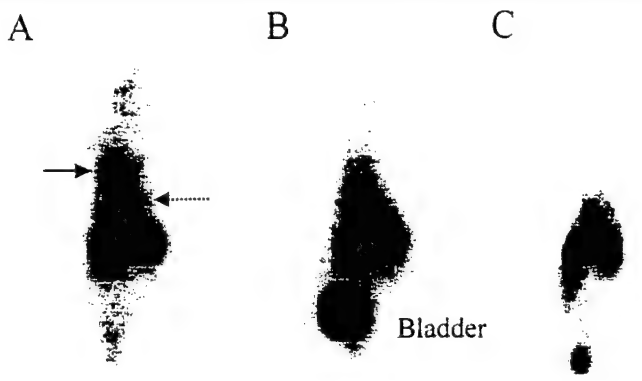

Figure 1. Biodistribution and clearance of Tc-99m full length AFP in a SCID mouse bearing a 0.05 gram human breast cancer (T-47D) in the left axillary region. The mouse was imaged with a very small tumor to assess biodistribution with little interference from tumor. Images A, B, and C are obtained at 8, 60 and 1440 minutes (24 hours), respectively, after dose administration. Immediate blood pool activity is evident in the heart region (solid arrow) which fades rapidly and is nearly at background levels by 60 minutes. Persistent activity is evident in the liver (dashed arrow) and kidneys at both 1 and 24 hours. No bowel activity is evident and soft tissue activity is low at 1 and 24 hours. A slight asymmetry is evident in the left axillary region at the site of the tumor, which was just palpable (<3mm diameter).

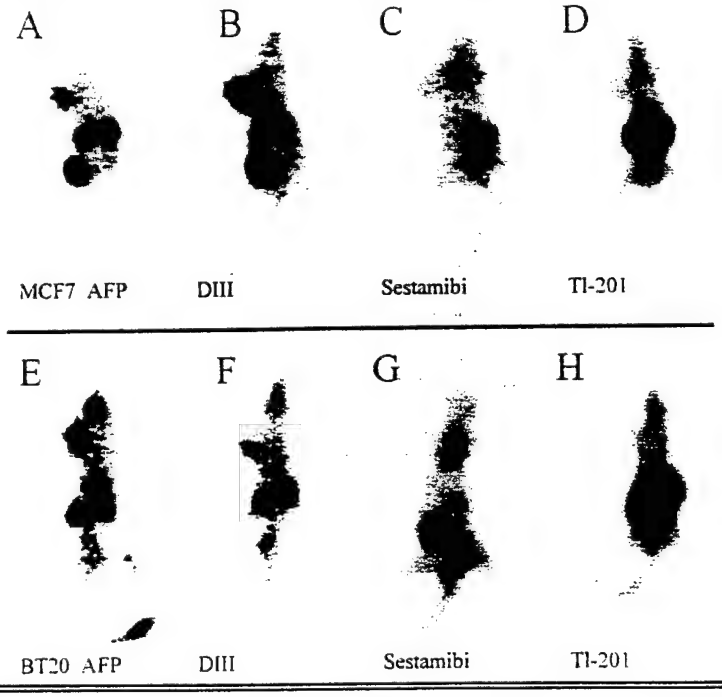

Figure 2. Images of human breast cancer xenografts in SCID mice bearing 1.0 to 1.5 cm diameter MCF7 tumor in the left axillary region obtained with Tc-99m AFP, Tc-99m DIII, Tc-99m sestamibi and T1-201, respectively, at weekly intervals in the same mouse (panels A-D respectively). Panels E-H show the same tracers in 0.5 cm diameter BT-20 human breast cancer xenografts. All images were obtained at 24 hours after tracer administration.

with breast cancer: Tc-99m sestamibi and Tl-201. The tumor size and appearance was recorded over the interval to incorporate size into measures of detectability and tumor to thigh tissue ratio. Tumor size ranged from 0.5 to 1.5 cm in diameter. Examples of imaging studies are shown in figure 2.

The image data were analyzed to address three specific questions. 1) Is radioactivity in tumor significantly above that in background thigh tissue? 2) Is radioactivity in tumor from Tc-99m AFP significantly above that from Tc-99m sestamibi or Tl-201? 3) Are there differences among tumors in radioactivity from Tc-99m AFP? To evaluate whether the activity in tumor is significantly greater than thigh tissue activity, we computed the ratio of counts per gram of tumor to the counts per gram of thigh tissue (T/Th). To compare levels of activity between tracers in a given tumor and for Tc-99m AFP preparations across tumors, the percent injected dose/gram of tumor (%ID/gram) was calculated. The quantitative comparisons of T/Th ratio and %ID/gram are shown in figures 3 and 4 and are detailed in Tables 1 and 2. This evaluation was performed for all tumors greater than 0.25 grams. Tumors smaller than this were excluded because they were difficult to locate reliably in the images. In the

T/Th results, values greater than one indicate higher tumor localization than thigh tissue activity. The tumor activity was compared to thigh background instead of the contralateral chest region, because, at the edge of the thorax, small changes in contralateral chest region placement produced highly variable target to background ratios. In all tumors studied, the Tc-99m AFP preparations yielded a T/Th ratio that was greater than 1, while Tc-99m sestamibi and Tl-201 yielded a T/Th ratio that was less than one. This was true both at 60 minutes and 24 hours after tracer administration.

When considering all tumor types together, ANOVA showed that DIII had significantly higher T/Th than AFP that, in turn, had significantly higher T/Th than both Tc-99m sestamibi or Tl-201. Because the tracers differed in their ability to detect different tumors (reflected as a significant interaction between tracer and tumor type) subsequent ANOVA with Student Newman Keuls multiple comparison

Table 1. Tumor to Thigh Tissue Ratios at 1 and 24 Hours.

Tumor to Thigh Tissue Ratios at 1 hour*

Tumor	AFP	Domain III	Sestamibi	Thallium
BT-20	[8] 1.30 (1.28)	[7] 3.35 (2.42)	[8] 0.24 (0.26)	[8] 0.55 (0.56)
MCF7	[4] 5.41 (1.84)	[6] 2.83 (1.80)	[8] 0.45 (0.43)	[8] 0.19 (0.27)
MDA-MB-231	[6] 0.56 (0.20)	[10] 1.57 (1.31)	[6] 0.41 (0.20)	[6] 0.15 (0.11)
MFE-296	[6] 3.54 (2.51)	[5] 8.50 (6.02)	[6] 0.71 (0.69)	[6] 0.49 (0.22)
MTW9A	[9] 3.58 (1.39)	[4] 4.62 (0.60)	[3] 0.81 (0.22)	[5] 0.65 (0.33)
T-47D	[4] 0.37 (0.47)	[11] 1.18 (1.43)	[3] 0.03 (0.14)	[3] 0.34 (0.21)

Tumor to Thigh Tissue Ratios at 24 hrs

Tumor	AFP	Domain III	Sestamibi	Thallium
BT-20	[8] 2.26 (2.18)	[7] 4.07 (3.33)	[8] 0.08 (0.11)	[8] 0.16 (0.20)
MCF7	[4] 4.70 (1.81)	[6] 5.33 (4.30)	[3] 0.26 (0.37)	[7] 0.21 (0.18)
MDA-MB-231	[6] 0.93 (0.61)	[12] 1.17 (1.00)	[6] 0.10 (0.08)	[6] 0.23 (0.08)
MFE-296	[6] 6.28 (1.60)	[5] 10.93 (5.58)	[6] 0.79 (0.49)	[6] 0.61 (0.11)
MTW9A	[9] 5.96 (4.17)	[4] 3.87 (0.75)	[3] 0.63 (0.26)	[4] 0.87 (0.17)
T-47D	[4] 1.80 (1.34)	[11] 3.90 (2.50)	[3] 0.27 (0.20)	[3] 0.03 (0.02)

*Data shown as number of animals in brackets, mean and standard deviation in parentheses

Table 2. Percent Injected Dose per Gram of Tumor at 1 and 24 Hours.

Percent Injected Dose per Gram of Tumor at 1 hour*

Tumor	AFP	Domain III	Sestamibi	Thallium
BT-20	[8] 0.98 (0.80)	[7] 2.24 (1.65)	[8] 0.11 (0.08)	[8] 0.43 (0.46)
MCF7	[4] 3.54 (1.79)	[6] 1.79 (1.15)	[8] 0.46 (0.62)	[8] 0.24 (0.35)
MDA-MB-231	[6] 0.27 (0.09)	[10] 0.62 (0.31)	[6] 0.17 (0.09)	[6] 0.21 (0.15)
MFE-296	[6] 1.47 (1.00)	[5] 2.96 (2.28)	[6] 0.42 (0.40)	[6] 0.34 (0.16)
MTW9A	[9] 2.70 (1.00)	[4] 2.79 (0.30)	[3] 1.31 (0.37)	[5] 1.63 (0.82)
T-47D	[4] 0.37 (0.49)	[11] 0.63 (0.82)	[3] 0.04 (0.18)	[3] 0.34 (0.18)

Percent Injected Dose per Gram of Tumor 24 hours

Tumor	AFP	Domain III	Sestamibi	Thallium
BT-20	[8] 0.44 (0.43)	[7] 1.37 (1.14)	[8] 0.02 (0.05)	[8] 0.16 (0.27)
MCF7	[4] 3.36 (2.09)	[6] 1.33 (0.89)	[3] 0.09 (0.14)	[7] 0.21 (0.20)
MDA-MB-231	[6] 0.19 (0.10)	[12] 0.28 (0.16)	[6] 0.01 (0.01)	[6] 0.21 (0.06)
MFE-296	[6] 1.36 (0.59)	[5] 1.86 (1.52)	[6] 0.15 (0.09)	[6] 0.51 (0.11)
MTW9A	[9] 1.04 (0.61)	[4] 1.75 (0.38)	[3] 0.24 (0.08)	[4] 1.49 (0.19)
T-47D	[4] 0.50 (0.30)	[11] 2.43 (2.26)	[3] 0.09 (0.04)	[3] 0.04 (0.03)

*Data shown as number of animals in brackets, mean and standard deviation in parentheses

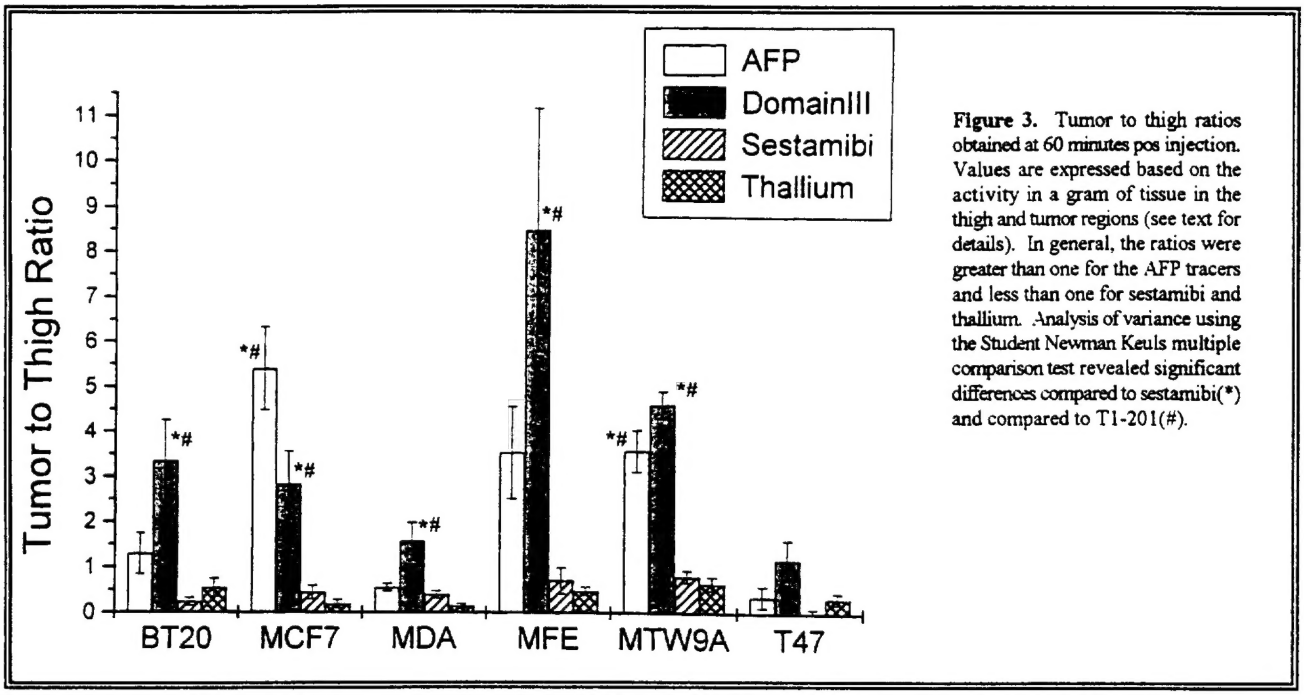

Figure 3. Tumor to thigh ratios obtained at 60 minutes pos injection. Values are expressed based on the activity in a gram of tissue in the thigh and tumor regions (see text for details). In general, the ratios were greater than one for the AFP tracers and less than one for sestamibi and thallium. Analysis of variance using the Student Newman Keuls multiple comparison test revealed significant differences compared to sestamibi(*) and compared to T1-201(#).

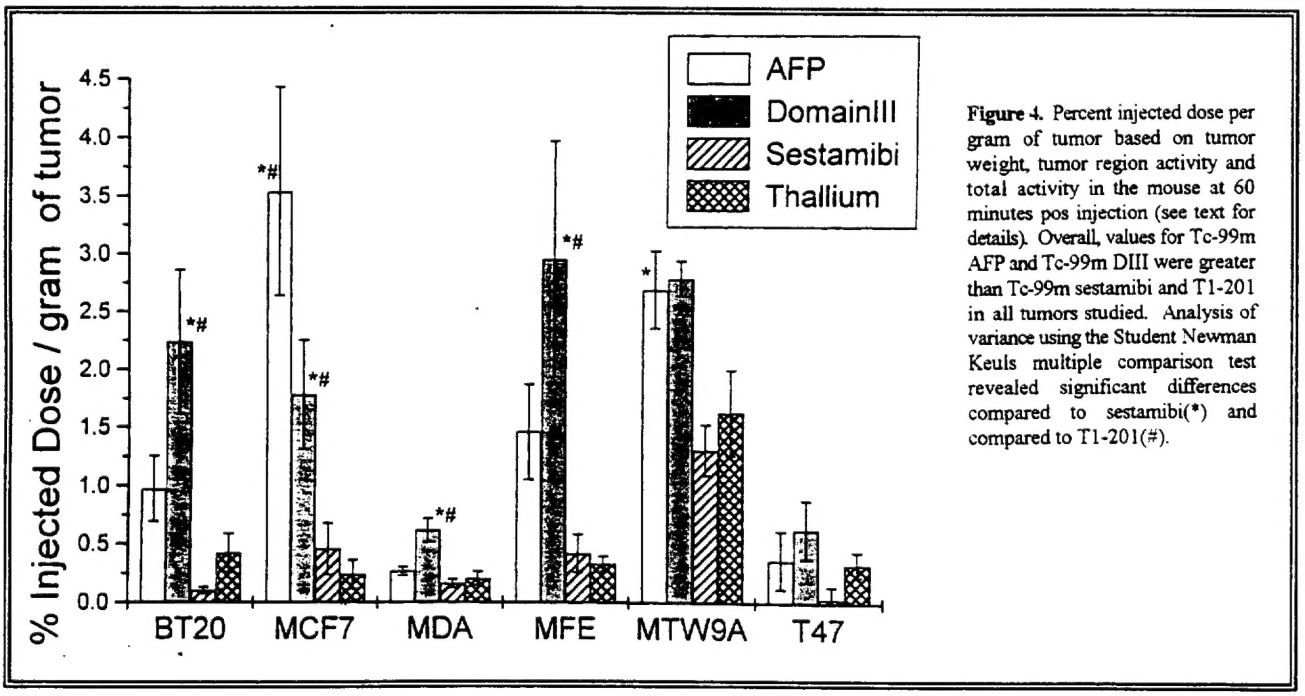

Figure 4. Percent injected dose per gram of tumor based on tumor weight, tumor region activity and total activity in the mouse at 60 minutes pos injection (see text for details). Overall, values for Tc-99m AFP and Tc-99m DIII were greater than Tc-99m sestamibi and T1-201 in all tumors studied. Analysis of variance using the Student Newman Keuls multiple comparison test revealed significant differences compared to sestamibi(*) and compared to T1-201(#).

tests were performed for each of the tumor types. Figure 3 shows 60 minute T/Th values which were generally lower than the 24 hour T/Th values, although there was little overall difference in the comparisons made at the two times. In most comparisons the T/Th measures are significantly greater for AFP and DIII relative to Tc-99m sestamibi and Tl-201 (see figure 3 for statistical significance of comparisons).

DIII and AFP had significantly higher percent injected dose per gram of tumor tissue than Tl-201 or Tc-99m sestamibi ($p<0.05$) when considered across all tumor types (Figure 4). This was true at both 60 and 24 hours after tracer administration. Using this parameter, there was no clear-cut difference in the imaging capability between radiolabeled full length AFP and DIII AFP. Figure 4 shows 60-minute percent-injected dose per gram of tumor,

which were generally higher than the 24-hour values. When considering individual tracers and tumors, %ID/gram for DIII was significantly ($p<0.05$) higher than Tc-99m sestamibi or Tl-201 in BT-20, MCF7, MDA-MB-231, and MFE-296 tumors (Table 2, Figure 4). The %ID/gram for AFP showed a similar trend but achieved statistical significance over Tc-99m sestamibi and Tl-201 only in the MCF7 tumor and over Tc-99m sestamibi in the MTW9A tumor. Thus, Tc-99m AFP and Tc-99m DIII appear to offer significant imaging advantages in a wide range of cell line tumors with regard to tumor uptake and relative contrast to normal tissue. MCF7 and T-47D are estrogen receptor positive whereas BT-20 and MDA-MB-231 are estrogen receptor negative, suggesting that hormone receptor status did not influence these results. Moreover, the estrogen receptor negative tumors grew more rapidly than the estrogen receptor positive tumors suggesting that growth rate did not influence these results. MFE-296 is a human endometrial tumor and MTW9A is a rat mammary cancer, suggesting that these findings may apply to other human and non-human cancers as well.

DISCUSSION

The results of this study demonstrate that AFP (natural or recombinant) labels efficiently with Tc-99m and localizes in human breast cancer xenograft tissue in vivo. One hour following i.v. administration of Tc-99m AFP, label is found primarily in the tumor and in the renal system. These results are in agreement with those reported by Uriel et al.,[13] who showed selective localization of murine I-125 AFP in syngeneic mouse mammary cancers in vivo. The mechanism by which AFP localizes in cancer cells may be via an AFP receptor described by Villacampa et al.[12] Malignant cells that have shown receptor-mediated uptake of AFP include human breast cancer cells, malignant lymphoblastoid cells, neuroblastoma cells, rhabdomyosarcoma cells and lymphoblastoid cells.[12,13,24] Although most dedifferentiated tissues have the ability to bind and endocytose AFP, this function is lost by differentiated cells.[12,25,26] The loss of this function in adult cells may be the basis for the selective tumor localization of AFP seen in this study. Our results support this speculation since AFP selectively localized in estrogen receptor positive as well as in estrogen receptor negative breast cancers, non-breast cancer (MFE-296) and a rat mammary cancer (MTW9A). These studies are the first to show selective human tumor localization of Tc-99m radiolabeled human AFP. The imaging capability of AFP compares favorably to that of Tl-201 and Tc-99m sestamibi, agents currently used to detect breast cancer in humans. Although results with Tc-99m sestamibi and Tl-201 have shown promise,[27-29] improvements in both sensitivity and specificity are needed. In our study, Tc-99m AFP had substantially better imaging characteristics for human breast cancer xenografts than did either Tl-201 or Tc-99m sestamibi. This finding, coupled with the fact that AFP is an endogenous human molecule with no known toxicity, strongly suggests that AFP should be developed further as an imaging agent for breast cancer. Both natural full-length AFP and DIII AFP were similar in their ability to image breast cancer in this study. For large-scale production, it seems reasonable to pursue recombinant systems and, in fact, such efforts are underway.[30]

Scintigraphic methods to detect breast cancer offer a means to improve the evaluation of patients with positive breast examinations or positive mammograms. Studies using radionuclides have suggested that scintimammography has high sensitivity and improves the specificity of conventional mammography. The conclusion of the study reported herein is that Tc-99m AFP images human breast cancer xenografts better than the scintimammographic agents, Tl-201 or Tc-99m sestamibi, currently in use. Thus, it seems desirable that Tc-99m AFP be developed further as a scintimammographic agent in an effort to improve on the early detection of breast cancer in women.

ACKNOWLEDGMENTS

The authors wish to thank Renita Singh and Daniel Bauer for their technical support. The United States Army (USAMRMC BC960165) and the American Cancer Society (ROG 398) provided funding for this work.

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